

IMMOBILIZED ANIMAL AND PLANT CELLS

PREPARATION AND POTENTIAL BIOTECHNOLOGICAL APPLICATIONS

BY

KJELL NILSSON



DEPARTMENT OF PURE AND APPLIED BIOCHEMISTRY

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To Eva,

Linnea, Olof, Marcus and Karin

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1. LIST OF PAPERS

This thesis is based on the following papers, which are referred to by Roman numerals in the text.

- I. K. Nilsson and K. Mosbach: Preparation of immobilized animal cells.
FEBS Letters, 118, 145 - 150 (1980).
- II. P. Brodelius and K. Nilsson: Entrapment of plant cells in different matrices.
FEBS Letters, 122, 312 - 316 (1980).
- III. K. Nilsson, S. Birnbaum, S. Flygare, L. Linse, U. Schröder, U. Jeppsson, P.O. Larsson, K. Mosbach and P. Brodelius: A general method for the immobilization of cells with preserved viability.
Submitted for publication.
- IV. P. Brodelius and K. Nilsson: Permeabilization of immobilized plant cells, resulting in release of intracellularly stored products with preserved cell viability.
Submitted for publication.
- V. K. Nilsson, W. Scheirer, O.W. Merten, L. Östberg, E. Liehl, H.W.D Katinger, and K. Mosbach: Entrapment of animal cells for the production of monoclonal antibodies and other biomolecules.
Accepted for publication in Nature.

2. INTRODUCTION

The general term 'immobilized cells' refers to the intentional physical localization of cells onto or within a support.

The use of immobilized cells is a natural development from the immobilized enzyme field. In the first reports on immobilized cells a single enzyme within the cells was used for production or transformations of biochemicals. By this way costly and tedious purification procedures were avoided and usually an enhanced stability of the enzyme was found as it was retained in its natural environment.

In the second generation of immobilized cells more complex reactions were carried out that required intact metabolic pathways and coenzyme regeneration. It was thus necessary to develop immobilization methods that resulted in preparations of cells with retained growth capability.

The main focus of immobilized cells has been concerned with microorganisms like yeast and bacteria. These species have a high growth rate and can create a considerable biomass in a short time thus diminishing the need for reuse of biomass which is one of the advantages with an immobilized cell system. Other advantages such as protection, ease of continuous operation, purer products and microenvironmental factors have created a considerable interest in immobilized cells. Since the first report of immobilized cells (Mosbach and Mosbach, 1966) the area has expanded very rapidly and in 1980 almost 200 reports appeared in the literature.

Most of the inherent advantages of immobilization can also be realized with the third generation of immobilized cells, immobilized animal and plant cells. As compared to bacteria and yeast they grow very slowly, which makes the generation of biomass expensive thus creating a need for its reuse.

Both plant and animal cells are sensitive to mechanical stress, plant cells due to their size and animal cells due to their lack of cell walls. The protection provided by immobilization thus facilitates their use in biotechnological processes.

The additional cost due to the immobilization process itself are negligible considering the high value at the complex products and in particular the fact that the production costs are diminished.

This thesis, based on paper I - V describes the preparation of immobilized animal and plant cells and their use for production of biochemicals.

3. IMMOBILIZED CELLS

3.1 GENERAL ASPECTS

The use of immobilized cells for production of biochemicals has several characteristics compared to a free cell system. These include:

Larger size of the biocatalyst.

Immobilized cell particles are about 10-1000 times larger than free cells. This makes the biocatalyst easier to handle. It allows the use of advantageous reactor designs such as packed beds, which are eminently suitable for continuous processes.

Confinement of cells to a support.

The product will be free of contaminating cells and the cells will be protected against mechanical stress to an extent depending on the immobilizing method.

The cells can also be coimmobilized with other cells or enzymes that enhances their catalytic activity. Inclusion of magnetic particles makes the catalyst easier to handle for instance in viscous media.

Microenvironmental factors.

The chemical composition of the support can be used to let the cells experience a favourable environment. Factors like hydrophobicity and charged groups are very important in the partitioning of substrate and products.

Reuse of the biocatalyst.

The easy reuse of the immobilized cells obviates expensive generation of biomass which is especially important with slow growing cells such as animal and plant cells.

With an immobilized cell preparation special attention has to be taken to diffusional limitations and intracellular products.

Diffusional limitations.

In contrast to surface bound cells the activity of entrapped and encapsulated cells are usually restricted by diffusional limitations. For example, in the interior of an immobilized cell particle, the cells are capable of consuming substrate at a faster rate than can be transported by diffusion. This diffusional limitation will be particularly pronounced

ced if the substrate has a low concentration in the bulk phase, due to low solubility, such as oxygen, or if it has a high molecular weight.

Even in conventional fermentors the necessary oxygen supply to dense cell populations is difficult to achieve. As immobilized cells can be used at even higher cell densities the oxygen supply may be insufficient. There exist several possibilities to improve oxygenation and in some of these the advantages gained by immobilization can be applied. Oxygen can be produced by illuminating algae. Thus coimmobilization of algae and bacteria has been used for enhancement of the oxygen supply (Adlercreutz et al., 1982, Wikström et al., 1982). Oxygen can also be generated by the decomposition of hydrogen peroxide. Thus, cells containing high catalase activity have been supplied with hydrogen peroxide thereby generating oxygen in situ (Holst et al., 1982). Hydrogen peroxide can also be degraded by manganese oxide or activated charcoal. Thus by coimmobilizing these catalysts with cells an enhanced production of alpha-keto acids was found (Brodelius et al., 1981, Szwajcer et al., 1982). In this case hydrogen peroxide was generated in the reaction leading to alpha-keto acid from the corresponding amino acid, thus obviating external supply of hydrogen peroxide.

The restriction of high molecular weight substances is highly dependent on the polymer structure. As shown in figure 4 cells entrapped in agarose will have access to proteins with a molecular weight of at least 150 000.

Intracellular products.

If the product is stored within the cells this limits the use of the biocatalyst in a continuous process. This is the case with most of the secondary products of plant cells. As shown in paper IV, these products can be released through permeabilization of the cells without affecting cell viability.

3.2 IMMOBILIZATION METHODS

An excellent review covering the area of immobilized cells is provided by Birnbaum et al. (1983). There are different routes to obtain an immobilized cell preparation:

Adsorption to a support.

This is the simplest of all immobilization methods. Cells and support are mixed and if the proper conditions are chosen, the cells will attach and stay at the surface of the support. The interaction between the cells and the support are usually a mixture of forces such as ionic bonding, electrostatic forces and/or hydrophobic bonding. The use of biospecific interactions have also been utilized, for instance human red blood cells have been reversibly immobilized on Concanavalin A-Sepharose and used in an analytical flow system (Mattiasson and Borrebaeck, 1978).

A special case of adsorption is the use of microcarriers for animal cell culture (section 4.2).

The main drawbacks of the method are low loading capacity (only the surface is utilized) and the risk of cell leakage (due to weak attraction forces). Since the cells are localized only to the surface, no protection against mechanical forces is provided. However, the diffusional limitations are minimized.

Covalent attachment to a support.

By introducing a chemical bond between the cells and the support, cell leakage can be minimized. However, this involves treatment with chemicals that usually affects cell viability negatively. This effect can be reduced by preactivation of the support before addition of cells, but still the method cannot be used in systems where cell growth is necessary. As for adsorption the diffusional limitations are small and no protection is provided. The preparation is also rather complex.

Ionic and covalently crosslinking

It is also possible to immobilize cells by treating a cell suspension with polymers (polyamines, chitosan etc). In this case the polymers forms bridges between the cells and thereby make them precipitate. Cell viability is usually high but the preparations show low mechanical stability and cell leakage frequently occurs.

The mechanical stability can be enhanced and cell leakage prevented by treating flocculated microorganisms with bifunctional reagents such as glutardialdehyde. Instead of using polymers, proteins like gelatin or albumin can be added to the cell suspension prior to addition of crosslinking reagent. By this way the precipitate will consist of cells covalently joined to each other, the added protein improves the mechanical strength. The covalent crosslinking reagent usually results in non-viable cells. As both ionic and covalently crosslinking to some extent resemble entrapment, diffusional limitations occurs.

Encapsulation

In order to confine the cells within a semi-permeable membrane, a variety of organic solvents and detergents, which are potentially dangerous to the cells are used. A water solution of the cells is emulsified in an organic solvent in which monomers or polymers are dissolved. The whole mixture is then emulsified in a water-phase, thus leaving the organic phase as a shell surrounding the cells. After polymerization the organic solvents are washed away.

The encapsulated cells are protected against mechanical forces, but depending on the membranes pore size, which usually is rather small, exclusion of high molecular weight substances frequently occurs. Due to diffusion barriers not all the cells will have access to optimal substrate concentration.

A more gentle method for encapsulation of animal cells has recently been described (Lim and Sun, 1980). This is discussed in section 4.3.

Entrapment.

Due to several advantages this method is the most commonly used.

The method has the following advantages:

1. The procedure is simple. Cells and polymer or monomers are mixed and upon gel formation the cells are encaged in a polymeric network.
2. The method is gentle. Because of the wide variety of polymers which can be used, a system can usually be chosen that retains the cells in a viable state.
3. The preparation exhibits decreased cell leakage. Due to the encagement leakage of cells is minimized.
4. It provides protection. The polymeric network protects the cells against mechanical forces.
5. The preparation has high loading capacity. The whole volume of the support can be used.

There exists also some disadvantages:

1. The preparation shows diffusional limitations. Not all cells will have access to optimal substrate concentration.
2. There will be excusion of certain substrates. The polymeric network is not usually penetrable to substrates of high molecular weight.

In principle, three different groups of polymers are used for entrapment; synthetic polymers, carbohydrates and proteins (Table I).

Miscellaneous

Cells can also be confined by using filters or membranes. One example is the use of hollow-fiber units for denitrification of water (Mattiasson et al., 1981).

An aqueous system, made up of polyethylene glycol and dextran, forms two phases above certain concentrations of the polymers. This property has been used for fermentation of glucose to ethanol by yeast cells. Yeast cells and glucose were added and the whole system was dispersed into microdroplets thus allowing an effective mass transfer. By ceasing the agitation two phases were formed with the cells confined in the lower dextran phase thus allowing product recovery from the upper phase (Kühn, 1980).

Table I Polymers used for entrapment of cells.

Polymer	Principle of gel formation	Gel-forming procedure	Reference
Synthetic polymers			
Polyacrylamide	Polymerisation	Addition of initiators to a solution of monomers	Mosbach and Mosbach, 1966
Epoxy resin	Polycondensation	Epoxy and polyamino precursors are allowed to cure in spherical shape with the aid of ionotropic gelation by alginate	Klein and Eng, 1979
Polyurethane	Polycondensation	Reaction of polyisocyanates with water	Klein and Kluge, 1981
Carbohydrates			
Cellulose	Precipitation	Cellulose is dissolved in organic solvent and precipitated in water	Linko et al., 1980
Agar, agarose	Thermal gelation	Cooling of a polymer solution	Toda and Shoda, 1975
Kappa-carrageenan	Thermal gelation + ions	Cooling of a polymer solution	Takata et al., 1977
Alginate	Ionotropic	An alginate solution is dripped into a CaCl_2 solution	Kierstan and Bucke, 1977
Proteins			
Collagen	Neutralisation	Neutralisation of an acidic solution	Elsdale and Bard, 1972
Gelatin	Thermal gelation	Cooling of a polymer solution	Gianfreda et al., 1980
Fibrin	Enzymatic	Enzymatic conversion of fibrinogen to fibrin	Pohland and Christophers, 1979

3.3 ENTRAPMENT OF CELLS IN BEADED POLYMERS

Entrapment of cells in the polymers listed in table I except kappa-carrageenan, alginate or epoxy gels usually results in a homogenous block which had to be fragmented to obtain a desirable size of the biocatalyst. This fragmentation results in loss of cells, irregularly formed particles with a decreased mechanical strength and usually a broad size distribution, the latter leads to poor flow characteristics in packed bed reactors. These disadvantages are avoided if the biocatalyst is prepared in a spherical form with a uniform size.

With alginate and kappa-carrageenan spherical beads are readily obtained by dripping the polymer solution into a salt solution (CaCl_2 and KCl respectively).

Spherical beads have also been prepared from epoxy and polyamino precursors. In this case alginate was used as a copolymer, the mixture was dripped into calcium chloride and after curing of the epoxy resin a porous structure was obtained, when the calcium alginate was dissolved (Klein and Eng, 1979).

But for the other polymers a two-phase system is necessary for bead formation. This two-phase system is based upon a water-immiscible solvent in which the polymer solution is dispersed by mechanical means. A mixture of toluene and chloroform is usually used. The method was originally developed for entrapment of enzymes in polyacrylamide (Nilsson et al., 1972) to which these solvents are not harmful. Attempts to use it for entrapment of cells usually results in a non-viable preparation.

The use of other solvents has also been reported, Klein and Schara (1980) used dibutylphthalate in the preparation of polyacrylamide beads with entrapped cells. The cells retained 90 - 100% of their phenol-degrading activity compared to free cells, but their ability to grow was not investigated. They also pointed out the importance of temperature control during polymerization. Block polymerization carried out at 25 - 30°C yields a much lower activity than cells entrapped in polyacrylamide through suspension polymerisation at 0 - 5°C. In a two-phase polymerization system, the heat generation during polymerization of for instance acrylic monomers, is easily diverted and isothermal conditions can be achieved.

Spherical particles of polyurethane have also been made in a two-phase system. In this case the solvent was liquid paraffin (Klein and Kluge, 1981). Also in this case no attempts were made to see whether the cells were capable of growing or not.

Most of the solvents used in earlier investigations are highly toxic and unsuitable for living cells. An alternative method to entrap cells in beaded polymers was therefore developed (paper III). The method is outlined in figure 1.

Choice of entrapping system.

1. Agar and agarose

This polymer shows hysteresis which means that it will dissolve at a higher temperature than the gel forming temperature. This property makes it useful for entrapment of cells. By chemically modifying its structure different gelforming temperatures are obtained. They are thus available in qualities that form gels at such low temperature as 15°C. There are, therefore, no restrictions in using these gels in combination with heat-labile species.

The polymer is dissolved by heating in a buffer that is compatible with the cells, and if necessary sterilized by autoclaving. When the polymer solution has been thermostated 5-10 °C above its gelforming temperature, it is mixed with cells.

2. Kappa-carrageenan

This polymer shows similar behaviour as agar but has also an ionic requirement. Thus the polymer is essentially insoluble in the presence of potassium ions. Due to the association of potassium ions to the polymer it has to be dissolved in a sodium chloride solution in which the sodium ions partly counteract the influence of potassium.

Chemically modified kappa-carrageenan with gelforming temperatures down to 15°C are commercially available.

A carrageenan sodium salt solution is thermostated at 5 - 10°C above its gelforming temperature and mixed with cells.

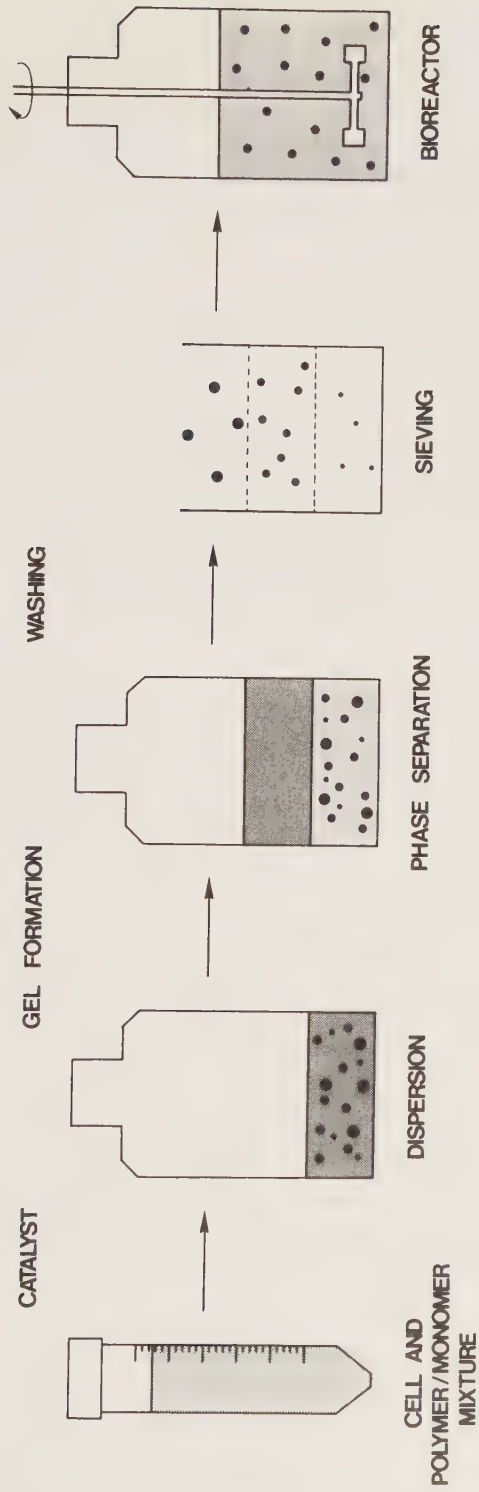


Fig. 1 Entrapment of cells in beaded polymers.

3. Polyacrylamide

In this case the polymeric network is built up from polymerization of monomers. The monomers are usually acrylamide and N,N -methylene-bis-acrylamide but a wide variety of different monomers is available. The monomers are brought to polymerize by addition of ammonium-persulphate as initiator and N,N,N',N'-tetramethyl-ethylene diamine (TEMED) as coinitiator.

Instead of mixing cells, monomers and initiators before dispersion in the hydrophobic phase (Nilsson et al., 1972, Klein and Schara, 1980) only one of the initiators is added together with cells and monomers in this work. The coinitiator, TEMED, is added when a desirable bead size is reached. This modification facilitates control of bead size as the polymerization proceeds very rapidly when both initiators are present. The monomer solution is steril filtered if necessary before mixed with cells.

4. Fibrin

Fibrinogen is enzymatically converted to fibrin by thrombin. The fibrin formed is water-insoluble and can be used to entrap cells. The cells are thus mixed with a fibrinogen solution (steril filtered) and thrombin is added before dispersion in the hydrophobic phase.

Formation of spherical droplets in a hydrophobic phase.

The water phase, containing the entrapment system, is dispersed in a hydrophobic phase by mechanical means. For small scale preparation a beaker and a magnetic stirrer is sufficient. The ideal hydrophobic phase should be cheap, non-toxic, easily disposable and easy to separate from the water phase. In order to compare different solvents agar-entrapped plant cells were chosen as a model system as these cells are sensitive to chemical reagents. A convenient measure of the viability was obtained by monitoring the respiration of the immobilized cells with an oxygen electrode. The results are summarized in Table II.

Table II. Relative respiration of agar-entrapped plant cells after immobilization in various hydrophobic phases.

Hydrophobic phase	Relative respiration (%)
Soy oil	100
Paraffin oil	91
Silicon oil	100
Tri-n-butylphosphate	100
Dibutylphthalate	82
Toluene:chloroform (73:27)	
+ Arlacel 83 (2% w/w)	0

As can be seen all solvents except the mixture of toluene, chloroform and detergent leaves the cells in a viable state with reference to respiration.

Subsequently soy oil and paraffin oil were further tested with different polymers. With agar, agarose and kappa-carrageenan the method works well but with fibrin and polyacrylamide a non-ionic detergent (Triton X-100) has to be included in the oil phase. The detergent is used in such a low concentration (0.05 % w/v) that no sign of toxicity appears. If the detergent is omitted, residues of the oil phase adhere to the beads and make them stick together.

Either soy oil or paraffin oil can be used as a hydrophobic phase, except for the entrapment of animal cells, when paraffin oil extracted with phosphate buffered saline has to be used. When soy oil or unextracted paraffin oil are used in combination with animal cells, the cells are irreversibly affected and no sign of cell growth can be detected. This effect is probably due to water-soluble toxic components, which in the case of paraffin oil are easily removed by extraction.

The bead size can be controlled in several ways and some important variables are:

- Power input
- Design of dispersion vessel.
- Viscosity of the cell and polymer mixture.

Usually the dispersion is made by a magnetic stirrer, but if smaller beads are necessary ultrasonication can be applied. If the dispersion vessel is equipped with baffles, a more narrow size distribution can be achieved, the baffles also reduce the necessary power input as compared to a clean vessel. The viscosity of the mixture to disperse also greatly influences bead size. Solutions of carbohydrates have a relatively high viscosity and demand a higher power input as compared to solutions of acrylic monomers.

The ratio of water-phase to oil phase is usually 1:5 but as much as 1:1 can be used when agarose is dispersed. This, of course, reduces the costs connected with immobilization and makes the method economically attractive. The oil phase can also be recovered as it is easily separable from the water.

Gel formation.

Agar, agarose and kappa-carrageenan solidify when cooled below their gelforming temperature. During dispersion the oil phase is held at an elevated temperature 5-10°C above the gelforming temperature and when the desired bead size is reached the whole vessel is cooled in an ice-bath.

Polyacrylamide is solidified after addition of the second catalyst (TEMED). The time for gel formation is depending on catalyst concentration. Usually a concentration is chosen that starts the polymerization within a few minutes.

Fibrin is gradually formed after addition of thrombin. The reaction starts immediately and after a few minutes the discrete beads have enough mechanical strength to prevent coalescence. The reaction is dependent on enzyme concentration which is chosen in such a manner that the reaction is completed within 15 minutes.

Removal of the hydrophobic phase.

When the gel formation is completed a washing medium compatible for the cells is added which in the case of kappa-carrageenan also should contain potassium ions to enhance the strength of these beads. As the oil phase has a lower density it will form the upper phase and beads with entrapped cells will sediment to the medium. If necessary a

gentle centrifugation will increase the sedimentation rate. The oil phase and most of the washing medium are then aspirated and new medium is added. This washing steps can be repeated until the medium is essentially free from oil.

Fractionation by sieving.

If necessary the formed beads are easily sieved on metal screens or nylon nets, thereby eliminating all but the desired size fraction. This sieving enhance utilization of the beads in continuous reactors where small beads can plug the outlet filters.

Sterility

It should be emphasized that all steps in the bead forming process can be performed under sterile conditions, thus preventing infections. This is a necessity when working with slow growing organisms such as animal and plant cells.

In Table III are summarized different species that have been immobilized and tested for production with this method. It can be seen that the methods works with bacterial, yeast, plant, algal and animal cells.

Table III Production of biochemicals by various cells entrapped in beaded agar or agarose.

Species	Product	Reference
Bacterial cells		
<i>Mycobacterium</i>	$\Delta^{1,4}$ -androsteradiene-3,17-dione	Paper III
<i>Gluconobacter oxydans</i>	2-ketogulononic acid	Paper III
<i>Pseudomonas putida</i>	2-ketogulononic acid	Paper III
Yeast cells		
<i>Saccharomyces cerevisiae</i>	Ethanol	Paper III
Plant cells		
<i>Catharanthus roseus</i>	Ajmalicine	Paper IV
Algal cells		
<i>Chlorella vulgaris</i>	Oxygen	Wikström et al., 1982
Animal cells		
MLA 144 (lymphoblastoid)	Interleukin 2	Paper V
LSP 21 (hybridoma)	Monoclonal antibody	Paper V
Genetically engineered cells		
<i>Bacillus subtilis</i>	Rat proinsulin	Mosbach et al., in press

4. ANIMAL CELL CULTURE

Animal cell culture can be used in several ways in practice: production and biosynthesis, toxicity and drug testing, amniocentesis, or therapeutic drug selection.

Production and biosynthesis

Animal cells synthesize many complex macromolecules of medical interest which, because of their complexity, cannot be chemically synthesized. At present, the only available source of these chemicals is animal tissues and in the case of chemicals unique to humans, the only source is human cadavers. If these products are produced in sufficient quantities by cells in culture, this could provide a convenient and continuous supply. Since most of the animal products of interest are synthesized by specialized cells, it is essential that cell lines can be derived from appropriate tissue and that they retain the properties of the original cells in culture. Some specialized cells grow very poorly in culture. Tumour cells, derived from them, will often grow better and are sometimes anchorage-independent which greatly facilitates large scale culture. However, the use of tumour cells is not allowed in the production of chemicals for human use, because they could contain carcinogenic agents. This legislation will probably be changed and it should be enough to prove that the product can be used in a safe way.

Up till now the most important commercial use of animal cell culture has been in the production of viruses for the preparation of vaccines. Virus will only grow in living cells and cell culture methods offer distinct advantages to the alternative methods which involve growth of viruses in whole animals or eggs. In Table IV are summarized the main products that can be produced by animal cell culture.

Table IV Products made from animal cells

1. Vaccines - polio, foot and mouth disease, rubella, measles, mumps, yellow fever, rabies
 2. Hormones - ACTH, prolactin, growth hormone, gonadotropin, prostaglandins
 3. Enzymes - plasminogen activator, collagenase
 4. Monoclonal antibodies
 5. Interferon
 6. Lymphokines
-

Toxicity and drug testing

The increasing concern about the harmful effect of chemicals in everyday use, such as food additives and cosmetics, has led to a tightening of control over the use of such chemicals. This necessitates stringent testing of any material for human use, and most of these tests are performed on laboratory animals. The use of alternative systems for some tests is desirable for many reasons, including saving on the numbers of animal needed, increased speed of testing and increased reproducibility. Cell culture could, in many cases, provide a suitable method for a primary screening of chemicals with possible toxic effects.

Amniocentesis

Amniocentesis has become an important technique for the diagnosis of genetic abnormalities in utero. A sample of foetal tissue is obtained, cultured and the chromosome content examined. The cells can be grown in small flasks, as only a small amount of material is needed. If amniocentesis is performed early in pregnancy, this would permit termination, if abnormalities in the chromosome content suggest that this is necessary.

Biochemical analyses to detect inborn errors of metabolism can be undertaken by subculturing the cells until sufficient material has been accumulated.

Therapeutic drug selection

By removing tissue from a patient and growing the cells in culture, it is possible to carry out a variety of tests which could not be made on the patient. When the tissue is a tumour, then the effects of anti-tumour drugs can be tested to determine the most suitable one for treating the patient.

4.1. TECHNIQUES IN ANIMAL CELL CULTURE

The procedure to obtain a cell culture from a human can be divided in the following steps: preparation of single cells from a piece of tissue, initiation of a monolayer culture and transformation to obtain a suspension culture.

Preparation of single cells from a piece of tissue.

A small piece of the appropriate tissue is removed aseptically.

A single cell preparation is usually obtained by a combination of the following disaggregation techniques:

1. Physical disruption.
2. Enzymatic digestion.
3. Treatment with chelating agents.

Before treating tissues with enzymes and chelating agents they are usually cut up into very small fragments. Enzymes that have been employed to disaggregate tissues are, for instance, trypsin, collagenase, elastase, pronase and dispase. Certain tissues seem to require divalent cations, particularly calcium and magnesium, for their integrity. If these ions are removed by chelating reagents (citrate, EDTA) the tissue may disrupt very easily.

Initiation of a monolayer culture.

After disaggregation the released cells are collected by centrifugation and transferred to a plastic tissue flask, growth medium is added and the culture is left overnight in a CO₂-incubator at 37°C.

The viable cells will attach to the plastic surface and can be released and cultivated further after treatment with trypsin.

Eventual transformation to obtain a suspension culture.

The cells thus obtained are designated a primary cell line and have a limited life span. After a number of dividings, less than 70, the cells will start to die.

However, sometimes a primary cell line undergoes alterations and can be subcultured indefinitely. Such cell lines are called established cell lines. This transformation can either be spontaneous or induced by virus or chemical reagents and is accompanied by an abnormal chromosome number in the cells. Established cell lines have short doubling times, 12 - 20 hours. They can be grown to much higher cell densities than primary cell lines and most of them can be established in suspension cultures.

Culture methods for animal cells.

Animal cells grow in two basically different ways. Monolayer cells are anchorage dependent and require a solid surface for their replication, whereas suspension cells grow freely throughout the bulk of a culture. Suspension cells can be grown in the same equipment as bacteria if one bears in mind that animal cells are more fragile due to the lack of cell walls.

While the anchorage dependence of monolayer cells offers no problems in small scale, it does mean that for large-scale production an extensive surface is necessary for cell growth. Various systems have been developed to overcome the problem of the space needed to accommodate a large surface area.

Until recently, the most popular methods of large-scale production involved multiple glass or plastic bottles that were either kept static or rolled. The surfaces available for growth were only those of the inside of the bottles. Such systems are labour intensive and require both a large amount of space and specific handling equipment. A further disadvantage is the variation that can arise between different bottles in a batch since it is not practical to control pH, for example, in every bottle. Finally, the risk for contamination rises very rapidly as the number of units to handle increases. In order to overcome these problems more or less sophisticated methods of introducing a surface into a container have been developed. Surface may be included as plates, discs, trays, films or, alternatively, tubes, fibers and concentric cylinders have been assayed. Packed beds offer unlimited opportunities for ingenious packing materials such as 'jacks', spirals or Raschig rings. However, most work has focused on systems based on beads of glass spheres of about 3 mm diameter. But these static-bed reactors provide an inhomogeneous environment and are difficult to control. Visual inspection is also difficult.

The two most promising techniques for large scale culture of animal cells are the hollow fiber device and microcarriers. Cell growth in vivo is supported by a vascular network that continuously delivers nutrients and removes waste products. In an attempt to mimic this situation the hollow fiber unit has been used to support cell growth. The unit consists of a bundle of capillaries usually 150 pieces with a diameter of 0.3 mm, that are closely packed in a glass or plastic

cylinder. Cells are usually attached and grown on the outside while medium is circulated within the capillaries. In a different set up air and carbon dioxide were circulated through the capillaries while medium was perfused perpendicular to them. Cells were thus constantly immersed in fresh medium and the gaseous exchange was efficiently carried out (Feder and Tolbert, 1983). Because of the efficient exchange of medium and gases extremely high cell densities are reached (up to 100 million cells/ml) which is about ten times more than in conventional culture systems (Gullino and Knazek, 1979). Cells are exposed to minimal stress and can thus be used for an extended period of time with continuous production of biomolecules such as urokinase and angiogenic factor (Feder and Tolbert, 1983).

The drawbacks encountered with the method are mainly inhomogeneity and sterility. The cells finally form a mass between the individual fibrers thus preventing an even flow of medium and gas. Most of the hollow fibrers are sterilized chemically with ethylenoxide or formaldehyde, after which extensive washing has to be performed. This is a time consuming process and in large scale equipment difficulties to achieve a complete sterilization are likely to appear. The potential of the hollow fiber device is very high and if these problems can be solved, it will increasingly find use in biotechnological applications.

An alternative and very promising method involves the growth of monolayer cells in quasi suspension cultures. An ingenious solution to the problem of providing a massive surface area for growth of monolayer cells was first proposed by Van Wezel (1967). He demonstrated that it was possible to culture human cells by growing them on a suspension of positively-charged dextran beads, 100-200 μm size. The cells adhere to the beads and will eventually grow to cover the particles. The growth rates are similar to that found in stationary cultures, but are achieved in a three-dimensional homogenous system. Thus, 20 ml of these beads provides a surface area equal to 80 Petri dishes (10 cm diameter).

4.2. THE DEVELOPMENT OF MICROCARRIERS FOR ANIMAL CELL CULTURE.

In the first experiments a commercial available ion-exchange gel (DEAE-Sephadex A-50) was used as a microcarrier (Van Wezel 1967). The main problem with this system was the toxicity of the beads when used at high concentrations. This toxicity was manifested by the failure of many types of cells to survive the early stages of culture, long lag periods and limited cell yields at the plateau stage of the culture. The toxicity could be partly counteracted by coating the beads with nitrocellulose or serum (van Wezel, 1973). Levin and co-workers found that by neutralizing some of the positively charged sites on the bead surface with a polyanion (carboxymethyl-cellulose) the toxicity was less pronounced and they were successful in growing a variety of cell types. They developed this concept further by synthesizing a series of beads with different charges (Levin et al., 1979) and found that cell growth is optimal within a limited range (1.5 - 2.0 mequiv/g dextran) which is much lower than DEAE-Sephadex A-50 (3.5 mequiv/g dextran). This optimized microcarrier showed no tendency to destroy the inoculum, caused no significant lag phase, and allowed cell growth up to high concentrations.

Optimal charged microcarriers based on dextran (CytodexTM₁, Pharmacia Fine Chemicals, Sweden and SuperbeadsTM, Flow Laboratories, USA) or polyacrylamide (BiocarriersTM, Bio-Rad Laboratories, USA) are now commercially available. Of these Cytodex and Superbeads, which are almost equal, have been proven successful for the culturing of over more than 100 different cell types. Up till now only a few reports have occurred about the use of Bio-carriers, and in one of these (Morandi et al., 1972) a comparison of the culturing of human MRC-5 cells was made between Cytodex, Superbeads and Bio-carriers. Cytodex and Superbeads turned out, as expected, to be equally good, while Bio-carriers produced significantly lower cell yields. This probably means that it is not only the surface charge which determines the cell growth but also the nature of the surface.

Commercially available plastic material (polystyrene) for cell culture has a negative charge, consequently this material has been prepared in spherical shape and used as a microcarrier (Biosilon^R, A/S Nunc, Denmark). Also for this microcarrier an optimal charge density was found (Johansson and Nielson, 1980).

Another type of supporting material for culturing of cells is represented by collagen and gelatin. Collagen would seem to be, almost functionally, the ideal choice since it is the natural substrate for cells.

Collagen has also been shown to enhance the growth as well as the differentiation of many cells in culture compared to other substrates such as glass or plastics (Kleinmann et al., 1981).

The use of collagen as a matrix usually employs reconstitution of collagen fibrers from an acidic solution of rat tail tendon collagen through evaporation, neutralization, chemical crosslinking or heating. The matrix is usually reconstituted as a film in Petri dishes.

The attachment of cells to collagen is mediated by proteins, one of these attachment proteins, fibronectin, is produced by certain cells such as fibroblasts and endothelial cells, it is also present in high concentrations in blood and serum (Mosesson and Armani, 1980).

The fibronectin-mediated attachment of cells to collagen proceeds in three steps (Kleinmann et al., 1981):

- a. fibronectin binds to collagen
- b. cells will bind to the collagen-fibronectin complex
- c. cell spreading will require fibronectin and cellular metabolism.

The binding of fibronectin to denatured collagen proceeds at a much higher rate than the binding to native collagen (Johansson and Höök, 1980) also the bound amount is higher (Jilec and Hörmann, 1978). Indeed a widely accepted method for purification of fibronectin involves affinity chromatography on gelatin covalently bound to Sepharose (Engvall and Rouslahti, 1977).

In order to mimic cell attachment in vivo a microcarrier based on gelatin was prepared (paper I). Gelatin was used as the cell attachment is equally good or even better than to collagen. Cheap raw material and a high solubility in neutral solutions are further advantages. Preparation of gelatin microcarriers is also extremely simple, a gelatin solution is dispersed in a hydrophobic phase and when the desired size distribution is reached the whole mixture is cooled resulting in solid beads. The hydrophobic solvent is washed away with acetone and after transfer to water cross-linking with glutardialdehyde is carried out. Excess reagent is washed away and after drying and sieving the beads are ready for use.

This microcarrier was tested with two anchorage-dependent cell cultures, S 157 N (human skin fibroblast) and DMH W1073 (rat colon carcinoma). As shown in Figure 2 both cell types attached and proliferated on the beads.

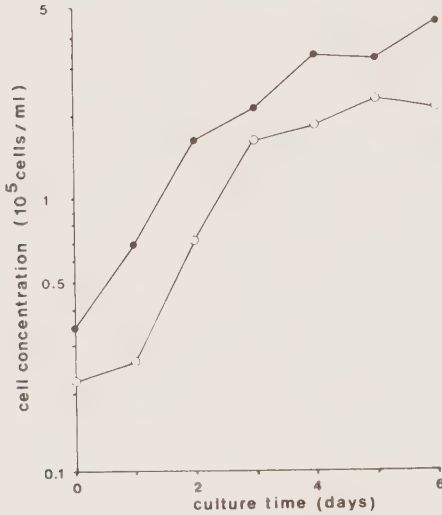


Fig. 2 The growth of two primary cell cultures, S 157N (●—●) and DMH W1073 (○—○), on gelatin beads.

No sign of toxicity, such as loss of inoculum and long lag periods, was observed for any of the tested cell types.

A particularly interesting property of this new microcarrier is the superior procedure for cell release. In figure 3 the result from three different enzymic procedures leading to cell detachment from gelatin microcarriers is given.

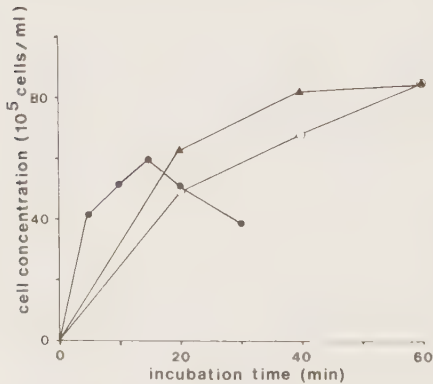


Fig. 3 Release of microcarrier-bound cells using trypsin (●—●), collagenase (○—○) and dispase (▲—▲), respectively.

As seen, both collagenase and dispase give rise to a far higher total cell concentration than normally applied trypsin treatment. In contrast to trypsin treatment which involves destruction of the cell surface to accomplish cell removal, dispase and collagenase dissolve the beads. This should leave the entire cell intact and viable which besides a higher yield is an advantage when the cells are used for immunization of when analysis of surface antigens will be done.

Recently, gelatin-coated dextran beads have been produced for animal cell culture (Cytodex 3, Pharmacia, Sweden). These beads will probably exhibit the same properties as the homogenous gelatin beads described above.

The gelatin microbeads also function as affinity material in cell separation. The biospecific interaction between gelatin and anchorage dependent cells was thus used in the separation of mononuclear cells from peripheral blood (Sjögren et al., in press). The gelatin beads were thus packed in a 10 ml syringe equipped with a 100 μ m nylon net and outlet flow control. After washing the column with phosphate buffered saline the beads were saturated with fibronectin by passage of medium containing autologous plasma and heparin. Heparin was added to enhance fibronectin binding to gelatin (Jilec and Hörmann, 1979) and is also known to potentiate binding of fibronectin to macrophages (Hörmann and Jelinic, 1980). When a mixture of Ficoll-Isopaque separated mononuclear cells are passed through the column, the non-adherent lymphocytes are recovered in the first fraction which is completely free of monocytes. An enriched monocyte fraction can be eluted with EDTA and further used as feeder cells required in hybridoma cultures (Brodin et al., in press).

The method is very rapid and the separated cells have excellent viability. The capacity is also very large, a 10 ml column can separate more than $250 \cdot 10^6$ mononuclear cells.

4.3. IMMOBILIZED ANIMAL CELLS

Microorganisms, such as bacteria and yeast, are easily cultivated in large scale. This is due to simple nutrient requirement, usually only glucose and some mineral salts are needed, and stability of the cells. Each cell is encaged in a tough cell wall which protects against mechanical forces. Each cell has furthermore its own metabolic machinery which can synthesize all complex molecules that are needed.

Animal cells, in contrast are adapted to live within a complex organism where each cell type has its function and are dependent on other cell types for its survival. When these cells are removed from the organism an extreme stable environment has to be provided. Variables that must be carefully controlled include temperature, pH, oxygen and carbon dioxide levels and osmotic pressure. Medium for animal cell culture includes salts, glucose, amino acids, vitamins and hormones. Still, they cannot support cell growth unless blood serum is added. A further complication arises because of the requirement of a surface to support cell growth for the majority of cells. These factors have hampered the use of animal cell culture for production of biochemicals.

Some of these problems have been partly solved. Anchorage dependent cells can be cultivated on a relatively large scale either by the use of hollow fiber units or microcarriers. However, the fragility of the cells fragility is still a major problem. A possible way to protect the cells is to provide them with an artificial "cell wall". This has been done either by entrapment or encapsulation of animal cells in polymers. Due to the sensitivity of the cells and their requirements of macromolecules (provided by addition of blood serum), a number of factors have to be considered in the choice of immobilization method.

The ideal immobilization method should be gentle and not involve any toxic chemicals. It should also be easily carried out under sterile conditions. The polymeric network should preferably allow free diffusion of macromolecules. As a consequence of the difficulties encountered with preparation of immobilized animal cells, only a few reports have appeared in the literature.

Natural gelforming polymers, fibrin and collagen, have been used to prepare films with entrapped cells. A number of different cell types, anchorage-dependent as well as suspension cells, have been entrapped in collagen by a simultaneous rise in pH and ionic strength of an acidic collagen solution (Elsdale and Bard, 1972). The cells survived and were able to grow within the gel lattice, and in the case of anchorage dependent cells, the regenerated collagen bundles supported attachment.

Soluble fibrinogen can be enzymatically converted to fibrin by thrombin. The fibrin formed is insoluble and has been used for entrapment of animal cells. Also in this case anchorage dependent cells attached to the fibrous network and were able to grow (Pohl and Christopers, 1979). The growth rate was dependent on polymer concentration. With 0.03% fibrin doubling time was 1.5 days in the exponential growth phase, compared to 7 days in 1% fibrin. It was suggested that the retardation in cell growth was due to nutritional deprivation.

These investigations proved that it was possible to entrap animal cells. However, their intended use was cell behavioral studies such as morphology, motility, adhesion and growth and the preparations were not suitable for biotechnological applications.

The alginate method has been employed for the immobilization of human red blood cells (Pilwat et al., 1980). Red blood cells and alginate were mixed and added dropwise to a solution of calcium chloride (10 mM). If higher concentration of calcium chloride was used release of hemoglobin occurred due to membrane damage. The immobilized cells retained their form for more than 5 weeks as judged by size distribution and microscopic observations. The method was only used for storage of red blood cells and no attempts were made to extend it to other cell types.

A gentle microencapsulation method, which avoids organic solvents and detergents, has been used for immobilization of animal cells. Cells are mixed with alginate and the suspension is added dropwise to a calcium chloride solution. The formed beads are treated with polylysine and polyethyleneimine, as polylysine and polyethyleneimine are both positively charged and have high molecular weights, they can only interact with carboxyl groups in the alginate bead's outer shell.

Finally the beads are treated with sodium citrate which liquify the alginate inside the capsule (Lim and Sun, 1980). By stabilizing the alginate capsules with positively charged macromolecules, the presence of calcium ions is not necessary to keep the network intact and it is also possible to use phosphate containing medium. In this way red blood cells, hepatoma cells, sperm cells, pancreatic endocrine tissues and pancreatic islets were immobilized (Lim and Moss, 1981).

Encapsulated hepatoma cells appeared to grow at about the same rate as freely suspended cells and had after 10 days grown to such an extent that the capsule burst.

Encapsulated islets were found to be more stable than free islets, which after 24 days showed a high level of base-line insulin release and an abnormal response pattern upon stimulation. The encapsulated islets, on the other hand, had a reasonable low base-line insulin release and a typical response pattern.

Further studies of encapsulated islets (Lim and Sun, 1980) demonstrated their potential for correction of diabetes. Implantation of encapsulated islets into diabetic rats reduced the symptoms for almost three weeks, while free islets survived only for 6 to 8 days.

Encapsulation thus proved to be a way to circumvent immune rejection. The method has also been used for production of monoclonal antibodies by encapsulated hybridoma cells (Editorial, 1982). The produced immunoglobulines are retained within the capsule and are released by addition of heparin which dissolves the capsule. Released antibodies are stated to be purer than those conventionally produced by tissue culture methods.

Isolated cells as well as cell cultures have also been entrapped in alginate and agarose (paper I). When isolated adipocytes were used to compare entrapment in alginate and agarose it was found that the alginate matrix exhibits a more profound negative influence on cellular behaviour than agarose. When attempts were made to cultivate alginate- entrapped cells the drawbacks of the method were even more evident. If medium with a high phosphate concentration was used, the beads showed a tendency to dissolve and precipitation, probably of calcium phosphate, appeared. These problems were of course, avoided when agarose was used as a matrix.

Cells were entrapped in agarose in two ways, either by mixing agarose with a small amount of alginate and subsequently adding the mixture dropwise to calcium chloride, (alginate was thus only used as an aid to prepare spherical beads and the main matrix consisted of agarose, these beads were thus stable in phosphate containing media) or by moulding cylindrical beads.

In order to obtain a desirable size and to avoid cell damage and cell losses due to fragmentation of a large block, cylindrical catalyst beads were moulded in a form made up from Teflon plates. This method could of course not be used for preparation of large amounts of biocatalyst but proved that agarose was suitable for entrapment of animal cells (paper I). Recent developments (paper III) resulted in an improved method to obtain spherical beads of agarose and other polymers which is suitable for large scale preparation of entrapped cells.

Anchorage dependent cells cannot attach to the polymeric network of alginate and agarose. Since attachment is necessary for cell growth inclusion of microcarriers was tested. As cell motility is restricted within the polymers, it was necessary to allow the cells to attach before inclusion of microcarriers. No clear proliferation of the entrapped cells was observed, but as judged by viability tests, the entrapment per se did not seem to damage the cells to any greater extent. Of the polymers tested agarose seemed to be the most promising.

As previously described (Pohl and Christophers, 1979) animal cells can be entrapped in fibrin with retained growth capability. However, as already has been pointed out, these preparations are not suitable for biotechnological application. By using the method described in paper III, spherical fibrin beads with entrapped cells could be produced. Due to the spherical shape a higher mechanical resistance was obtained and the preparation could be used in a continuously stirred system for more than one week without any sign of damage. Both suspension cells and anchorage-dependent cells were able to grow within the matrix and in the case of anchorage-dependent cells the fibrin fibers were used as the required surface. The decrease in cell growth that had been observed with high concentrations of fibrin (Pohl and Christophers, 1979) was not found with these spherical beads. This is probably due to

the more efficient exchange of nutrients that is obtained with suspended microbeads compared to the film previously used.

Production of these fibrin beads is also very simple. Cells are mixed with a fibrinogen solution and the mixture is dispersed in a hydrophobic phase after addition of thrombin. The only hydrophobic phase that has been found to be non-toxic is paraffin oil that has been extracted with phosphate buffered saline. Addition of a non-ionic detergent, Triton X-100, to the hydrophobic phase is necessary to facilitate transfer of the formed beads to medium.

The potential of using entrapped animal cells for production of biomolecules is demonstrated in paper V. As a model system, production of monoclonal antibodies and lymphokines by hybridoma cells and lymphoblastoid cells were used, respectively. Both these cell types are suspension cells and do not require attachment to a surface for growth. Cells were entrapped in agarose by the method described in paper III, but the hydrophobic phase was extracted paraffin oil.

Table V. Production of biomolecules by entrapped animal cells.

Day	Production of interleukin 2 by entrapped MLA 144 cells (titer)	Production of monoclonal anti- body by entrapped LSP 21 cells (reciprocal dilution).
1	1350	256
2	1670	512
3	1000	256
4	1480	512
5	1350	512
6	1350	n.d.
7	n.d.	256
8	950	n.d.
10	1500	n.d.
13	1440	n.d.

n.d. = not detected

The amount of antibodies and lymphokines produced were comparable to the production in a free cell system. When examined microscopically, it was found that the cells were able to grow within the beads, thus making it possible to use the biocatalyst for an extended period of time. As also demonstrated, cells could be entrapped in an extremely high concentration, that cannot be achieved by conventional cell culture methods. This means that a higher product yield per reactor volume and higher product concentrations can be achieved.

Of the three methods that have been successfully employed for entrapment of animal cells, microcapsules, fibrin beads and agarose beads, the latter seems to be the most promising for biotechnological applications (table VI).

Table VI Properties of immobilized animal cell preparations.

Properties	Microcapsules	Fibrin beads	Agarose beads
Mechanical strength	relatively low	relatively low	relatively high
Preparation	complex	simple	very simple
Suitable for anchorage-dependent cells	no	yes	no
Susceptible for proteolytic enzymes	no	yes	no
Release of high molecular weight products	no	yes	yes
Restriction in cell growth	no	no	yes
High cell densities	no	no	yes

The mechanical strength of agarose beads can easily be varied by changes in polymer concentration and is usually higher than for fibrin and microcapsules. The preparation is also very easy and can be performed under sterile conditions. Agarose and microcapsules are however not suitable for immobilization of anchorage-dependent cells but could probably be used after inclusion of materials that support cell attachment. Fibrin is readily attacked by proteolytic enzymes that are released from lysed cells.

The main drawback of microcapsules is the retainment of high molecular weight products inside the capsule. This obviates their use in continuous processes and could totally prevent their use, if the produced substance is toxic in higher concentrations.

Agarose is highly porous and allows high molecular weight substances to freely diffuse throughout its network.

In figure 4 the release of entrapped antibody (molecular weight 150 000) labelled with ^{125}I is monitored.

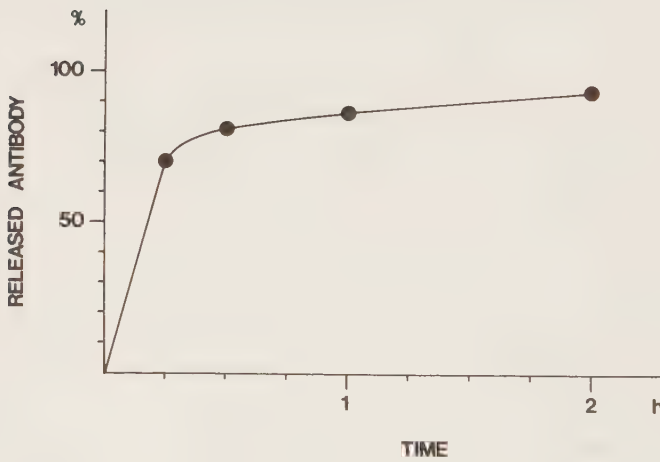


Fig. 4 Release of agarose-entrapped antibody.

The antibody was entrapped in beaded agarose (2 % w/v, 5 ml). After gel formation 15 ml phosphate buffered saline was added and the oil phase was removed. Samples of the water phase were taken periodically and assayed for radioactivity. Percent radioactivity in the water phase compared to the final equilibrium radioactivity for the whole system was calculated and plotted against time. During the first 15 minutes 70% of the entrapped antibody was released and after 2 hours the release was more than 90% (unpublished results).

Cells entrapped in fibrin or microcapsules usually grow to such an extent that the polymeric matrix disintegrates. Cell growth in agarose beads is restricted to some extent depending on polymer concentration. This is probably due to a mechanical influence of the polymeric chains. This restriction in cell growth makes it possible to use the biocatalyst over a longer period of time. It is also possible that this growth retardation changes the metabolism of the cells in such a way that medium consumption is more directed to product generation than to cell multiplication. It should therefore be possible to construct a simplified, and thereby cheaper, medium that promotes a higher product concentration. The steric hindrance may also be beneficial for stability of some hydrides which tend to lose chromosomes responsible for product generation, when they are in rapid proliferation. Extremely high cell densities within the beads can also be used which would cause microcapsules and fibrin beads to disintegrate.

5. PLANT CELL CULTURE

Aside from the primary metabolic pathways common to all life forms, some reactions lead to the formation of compounds unique to a few species. These reactions are classified under the term secondary metabolism and their products are known as secondary metabolites. Many of these secondary metabolites have a very complex structure and cannot be chemically synthesized in an economically feasible way.

In table VII the most common compounds derived from higher plants are listed. As much as 25% of all prescribed drugs in the US contained products of higher plants. The aroma compounds are mostly terpenoids which possess highly esteemed olfactory and taste properties.

Another group of high-priced compounds is the essential oils which are used in perfumes. The peptides thaumatin and monellin have zero calories and high power as sweetening agents. Pyrethrin has found widespread use as insecticide.

Table VII Compounds derived from higher plants (Zenk , 1978)

Pharmaceuticals	- Steroids, codeine, atropin, reserpine, hyocanine, digoxine, scopolamin, digitoxin, pilocarpine, quinidine, solcinine, emetine, morphine, quinine, sennosides, tubocurarine, vincristine
Aroma compounds	- (+)-nootkatone, valencene
Essential oils	- roseoxide
Sweetening agents	- thaumatin, monellin
Insecticides	- pyrethrin

These compounds are usually produced by conventional agriculture methods with all their inherent drawbacks such as:

- A. Production is dependent on climate, seasonal variations and soil quality.
- B. Uncontrolled production of important drugs like morphine and codein
- C. Association of microbes and insects with the plant material
- D. Large variations in the quality of plant material.

These disadvantages can be avoided if the compounds are produced by cell culture methods. In cell culture, secondary metabolites can be produced in two basic ways, either by de novo synthesis or from precursors. In de novo synthesis the cells are fed by nutrients and synthesize the desired compound (among several others) from a carbon source, such as sucrose. However, if the pathway for synthesis is known one can feed the cells with a precursor, which usually is much cheaper than the final product. In this way the yield of the desired compound can sometimes be increased considerably (Tabata, 1977).

The synthetic machinery of plant cells can also be utilized for chemical transformation of a compound - biotransformation. Plant cells contain enzymes and multi-enzyme systems which are not found in microorganisms. In this way high valuable compounds can be generated from low cost products or totally new compounds can be produced by adding analogs of natural substances.

5.1 TECHNIQUES IN PLANT CELL CULTURE

The procedure to obtain a cell culture from a plant can be divided into the following steps: preparation of a sterilized explant, formation of a callus culture and transferring to liquid medium.

Preparation of a sterilized explant.

A fairly large piece of tissue is taken from the plant. This explant may be pieces of stem, leaf, root, flower, fruit or seed. The explant is surface sterilized with, for instance, sodium hypochlorite and cut into smaller pieces.

Formation of a callus culture.

The sterilized explant is transferred to a medium solidified with agar. After about one month a callus is formed which consists of undifferentiated cells. Pieces of this callus are then transferred to fresh medium and this transferring is repeated until a more or less uniform tissue is obtained.

Transferring to liquid medium.

As only part of the callus is in contact with medium, gradients of nutrients, respiratory gases and toxic waste products will appear. The callus is therefore transferred to liquid medium and incubated on a gyratory shaker.

Medium requirements.

The basis for all nutrient media for plant cells is a mixture of inorganic salts together with a carbon source which is usually sucrose. Most plant cells also require addition of vitamins and phytohormones. Occasionally undefined organic nutrients such as coconut milk or protein hydrolyzate are added.

5.2. ENTRAPMENT OF PLANT CELLS

Entrapment of plant cells have several potential advantages:

- A. Protection against mechanical forces is obtained. Due to their size, plant cells are relatively sensitive to shear forces.
- B. Better reactor performance is achieved. Plant cells have a tendency to grow in aggregates (from a few cells up to several hundred cells, depending on the cell line), this makes it difficult to obtain a homogenous suspension of cells throughout the reactor. In an optimal immobilized preparation all the beads have approximately the same size, this means that the beads can be agitated with a minimum of power input.
- C. Continuous operation is easier. Because of their size, beads can easily be retained inside the reactor by means of a glass filter or a net.
- D. Beneficial microenvironmental factors may result. The cells have the opportunity to create their own environment inside the beads due to diffusional limitations.

Viability of immobilized cells.

The potential uses of immobilized plant cells is the production of very complex secondary metabolites and biotransformations. This requires that the cells are in a viable state. The standard procedure to measure viability is plasmolysis, respiration and cell growth.

Plasmolysis

By exposing the cells to osmotic stress, the integrity of its membrane can be observed. Glycerol or sorbitol are commonly used as plasmolyzing reagents. For convenience the uptake of a dye (eg. phenosaffranin) is followed. If the membrane has been damaged the dye will penetrate it.

Respiration

Measurement of oxygen consumption by cells gives information about any eventual damage to the respiratory chain. Oxygen consumption is conveniently measured with an oxygen electrode.

Cell growth

The ultimate criteria for viability is cell growth. Growth rate is usually determined by direct measurement of increase in cell dry mass. Differences in growth rates are not necessarily related to viability for immobilized cells. A factor that also influence cell growth is the physical and chemical nature of the polymers.

The mechanical strength is dramatically increased when polymer concentration is raised. This is due to a closer network of the individual polymer chains. This results in a bead with lower porosity and in which cell growth is retarded. Presence of charged groups, like in alginate and carrageenan, creates a microenvironment which differs from the surroundings in, for instance, pH. Charged molecules may also be attracted or repelled which causes either enrichment or depletion. This means that the culture media, whose composition has been carefully determined for optimal growth of cells in suspension, are not necessarily optimal for immobilized cells.

Preparation of immobilized plant cells.

For long term production it is necessary to activate the biocatalyst in situ. This is done by feeding nutrients and thereby initiating cell growth. However, with continuous cell growth, beads become crowded with cells and start to disintegrate. It is also known that for some secondary metabolites the production rate increases as cell growth decreases. The optimal use should therefore consist of a production phase in which cell growth is limited by exclusion of hormones or nutrients, and when the activity decreases a reactivation phase is initiated by addition of complete medium. When the activity is raised to a normal value, a new production phase is started and so on. It is therefore necessary to immobilize the cells in such a manner that cell growth is possible. The most widely used method is entrapment, but in one case (Jirku et al., 1981) covalent attachment was employed. The support, polyphenyleneoxide, was activated with glutaraldehyde and after washing away excess reagent, cells were added and allowed to react with the support. Nothing was however stated about cell viability and if cell growth was possible.

In the first report on entrapped plant cells (Brodelius et al., 1979) calcium alginate was used as a polymeric matrix. This is a simple and

gentle technique. Beads can easily be prepared under sterile conditions, which is very important as infections by microorganisms have to be avoided among the slow growing plant cells. This polymeric network has an absolute requirement for calcium ions, which means that cell release can be effected through addition of calcium binding reagents. In some cases this is an advantage (cell counting, viability tests), but it is also the main drawback of the method. To maintain bead stability calcium chloride has to be added to all media, usually at a concentration of 5-20 mM. Phosphate is usually employed as a buffering substance in media. This causes precipitation and bead instability due to formation of calcium phosphate.

To some extent this can be avoided by substituting phosphate with 2-(N-morpholino)ethane sulphonic acid as the buffering substance (Jones and Veliky, 1981a). This is however not a general method as phosphate may be essential to the metabolic activity of the cells.

To circumvent this problem a number of other polymers were tested (paper II). These include agar, agarose, carrageenan, gelatin, polyacrylamide and combinations of gelatin with agarose and alginate. Gelatin and its combinations were subsequently crosslinked with glutardialdehyde in order to stabilize the beads. In all cases when plant cells were exposed to glutardialdehyde irreversible damage to the respiratory chain resulted and no cell growth occurred. In the case of polyacrylamide, monomers and/or initiators were toxic and damaged the cell membrane. Cells entrapped in agar, agarose, carrageenan and alginate were viable and capable of growing. (Table VIII).

Table VIII. Comparison of various preparations of immobilized *Catharanthus roseus* cells.

Preparation	Plasmolysis	Respiration	Cell growth
Alginate	+	+	+
Carrageenan	+	+	+
Agar	+	+	+
Agarose	+	+	+
Gelatin	+	-	-
Polyacrylamide	-	-	-

Further investigations of the growth pattern of entrapped cells showed that cell growth in agar and agarose is approximately equivalent to the growth of free cells, while cells entrapped in alginate and kappa-carrageenan were retarded in their growth (paper III and figure 5).

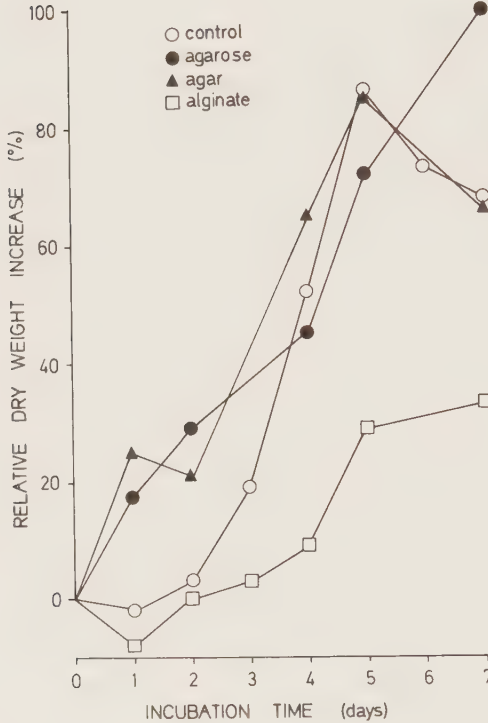


Fig. 5 Relative dry weight increase of various preparations of *Glycine max* cells as a function of incubation time. Control = freely suspended cells.

Agarose, which is a refined form of agar, fulfills almost all the requirements of an optimal polymer for entrapment of sensitive cells, such as:

- A. It is non-toxic.
- B. It has large pores which allow free diffusion of high molecular weight substances.
- C. It has no requirements of counter ions.
- D. It has no charges which would give unwanted ionic binding of substrates and products.
- E. It does not retard cell growth.

However, its mechanical stability can be too weak for some applications, such as in reactors with high shear forces.

Biosynthetic capacity of immobilized plant cells.

The biosynthetic capacity of plant cells can be divided into three categories: de novo synthesis, synthesis from precursors and biotransformations. Among these, biotransformations have been the most thoroughly studied for immobilized plant cells, as evident from table IX. The principle scheme for biosynthesis of ajmalicine is outlined in figure 6.

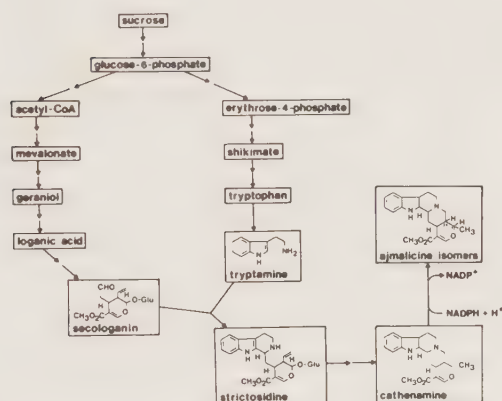


Fig. 6 Biosynthesis of ajmalicine.

De novo synthesis.

For the conversion of a simple carbon source (e.g. sucrose) into highly complex compounds, a large number of enzymes within the cells have to be working efficiently. Thus, the immobilized cells have to be in a viable state and preferably capable of growth. The de novo synthesis of a complex secondary product, anthraquinones, by alginate entrapped *Morinda citrifolia* cells was found to be ten times higher than by freely suspended cells (Brodelius et al., 1980). Also in the case of alginate entrapped *Catharanthus roseus* cells higher product yield (ajmalicine) was obtained compared to free cells (figure 7). With the same cells entrapped in agarose only a slight increase in product yield was found (paper II). The reason for this is not yet clear but obviously microenvironmental factors have an important part. Solubility

of oxygen is, for instance, dependent on ionic strength (lower solubility at higher ionic strength) which is higher inside the alginate beads due to the presence of charged groups and counter ions. As alginate is a negatively charged polymer (carboxyl groups) a lower pH inside the beads compared to the bulk solution results. De novo synthesis of steroid glycoalkaloids by covalently attached *Solanum aviculare* has also been described (Jirku et al., 1981).

Synthesis from precursors

This approach to biosynthesis requires knowledge of the biosynthetic pathways. By feeding the cells with an appropriate precursor they can be utilized more efficiently because of a higher substrate concentration than what is usually found within the cells can be used. Cells of *Catharantus roseus* can condense tryptamine and secologanine to strictosidine which, after a number of enzymatic reactions, yields ajmalicine as the end product (figure 6). This complex enzyme system was used to evaluate the usefulness of different polymers for entrapment of plant cells (paper II). As in de novo synthesis, alginate entrapped cells showed the highest productivity (figure 7). The power of precursor feeding is evident when the amount of ajmalicine produced with precursors is compared with de novo synthesis. With alginate entrapped cells twelve times more is produced in five days with precursors than for two weeks by de novo synthesis.

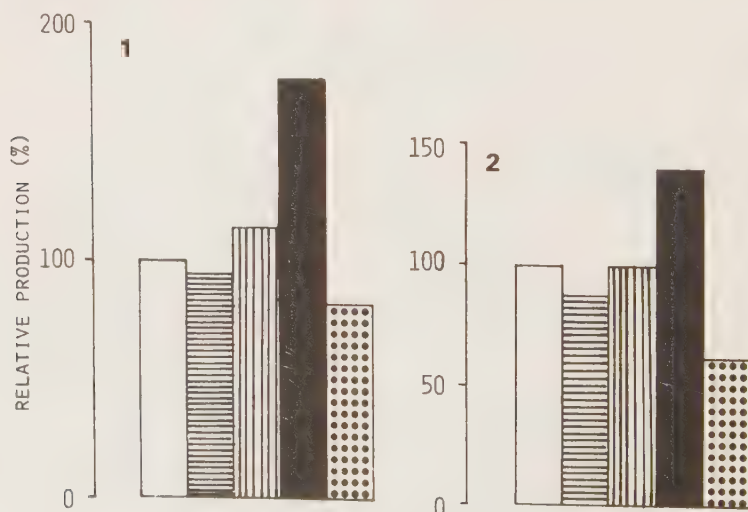


Fig. 7 Relative production of ajmalicine by immobilized cells of *Catharantus roseus* either from precursors, 1, or by de novo synthesis, 2. □ free cells. Entrapped cells : ■ agar, ▨ agarose, ■ alginate, ▤ carrageenan.

Biotransformations

Plant cells are able to perform specific chemical reactions on organic compounds. These compounds do not necessarily have to be natural intermediates in the metabolism of the cells. Biotransformations can be used for reactions such as: hydroxylation, glycosylation, dehydrogenation, methylation, carbon-carbon cleavage, ring opening, etc. Plant cell cultures have been screened for a large number of transformations (Reinhard and Alfermann, 1980). The potential of immobilized plant cells to carry out biotransformations have been demonstrated in a number of reports (table IX).

Conversion of digitoxin has been carried out by immobilized *Digitalis lanata* cells (Brodelius et al., 1979). The immobilized cells formed digoxin at approximately the same rate as a free cell system for a period of 33 days. With another strain of *Digitalis lanata* cells β -methyl-digoxin was formed from β -methyldigitoxin (Alfermann et al., 1980). In this case the immobilized cells were able to perform the reaction for more than 60 days. Activity of the immobilized cells was approximately 50% of the freely suspended cells. When this strain was tested for conversion of digitoxin to digoxin only small quantities were found. Instead most of the substrate was converted to purpureaglycoside A. This points out the importance of proper strain selection.

Immobilized cells of *Daucus carota* have been used for conversion of digitoxigenin and gitoxigenin (Jones and Veliky, 1981 a,b, Veliky and Jones, 1981). In this case the 5 β -hydroxylating activity of these cells was used. Conversion of digitoxigenin to 5 β -hydroxydigitoxigenin was studied batch wise and the biocatalyst could only be used for a few consecutive batches, probably due to the toxic effects of unreacted digitoxigenin within the cells. When a less toxic substrate, gitoxigenin, was used the biocatalyst could be used in a bioreactor for more than 30 days (Veliky and Jones, 1981). They also found that the mode of aeration had a profound effect on the transforming activity. When the cells were aerated by direct contact with air (air bubbling through the reactor) a higher conversion was achieved compared to aeration of only the medium (air bubbling through the medium only). They also used periodical activation of the cells with growth medium and could thus extend the biocatalyst half-life.

Table IX Biosynthetic capacity of immobilized plant cells.

Reaction	Immobilized species	Reference
De novo synthesis		
Ajmalicine	<i>Catharanthus roseus</i>	Paper II
Anthraquinones	<i>Morinda citrifolia</i>	Brodelius et al., 1979
Steroid glycoalkaloids	<i>Solanum aviculare</i>	Jirku et al., 1981
Synthesis from precursors		
Tryptamin + secologanin $\rightarrow$ ajmalicine	<i>Catharanthus roseus</i>	Brodelius et al., 1979, Paper II, Paper IV
Biotransformations		
Digitoxin $\rightarrow$ digoxin	<i>Digitalis lanata</i>	Brodelius et al., 1979
β -methyl digitoxin $\rightarrow$ β -methyl digoxin	<i>Digitalis lanata</i>	Alfermann et al., 1980
Digitoxin $\rightarrow$ purpureaglycoside A	<i>Digitalis lanata</i>	Alfermann et al., 1980
Gitoxigenin $\rightarrow$ 5 β -hydroxygitoxigenin	<i>Daucus carota</i>	Veliky and Jones, 1981
Digitoxigenin $\rightarrow$ 5 β -hydroxydigitoxigenin	<i>Daucus carota</i>	Jones and Veliky, 1981
Cathenamine $\rightarrow$ ajmalicine isomers	<i>Catharanthus roseus</i>	Felix et al., 1981

5.3. PERMEABILIZATION OF IMMOBILIZED PLANT CELLS

Plant cells generally tend to accumulate their secondary metabolites in vacuoles or in the cytoplasm. Usually these stored products are recovered by extraction of the cell mass with organic solvents. As one of the advantages with an immobilized system is a continuous operation, methods for release of these products have to be developed.

The cell membrane can be made permeable for low molecular weight substances by treatment with a reagent that interacts with its components. Reagents of this kind include, for instance, organic solvents, antibiotics, proteins and detergents (Felix, 1982). By feeding immobilized *Catharanthus roseus* cells with precursors and continuously extracting lipophilic products by bubbling the recirculated medium through chloroform, a high release of ajmalicine was achieved (Brodelius et al., 1979). Ajmalicine is usually stored within the cells but traces of chloroform in the medium resulted in a permeable cell membrane.

The fact that coenzymes such as ATP and NADP cannot penetrate an intact cell membrane can be used to monitor its permeability. The activity of intracellular coenzyme-requiring enzymes, such as hexokinase, malic enzyme, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase and citrate synthetase are assayed after addition of exogenous coenzyme, the amount of enzymatic activity thus found is a measurement of membrane permeability (Felix et al., 1981). The effect of a number of different permeabilization reagents on *Catharanthus roseus* cells have been compared (Felix et al., 1981). The relative enzyme activity of the two-enzyme system hexokinase/glucose-6-phosphate dehydrogenase was determined over a period of seven days. Maximum initial enzyme activity was found after treatment with dimethyl sulfoxide (DMSO), this activity was reduced to 20% after seven days. These permeabilized cells could also transform cathenamine to ajmalicine and release the product into the surrounding medium (Figure 7). In an attempt to stabilize the biocatalyst different matrixes and/or chemical reagents were employed (Felix and Mosbach, 1982). While isocitrate dehydrogenase activity was reduced to 50% after 12 days with DMSO-permeabilized agarose entrapped *Catharanthus roseus* cells, the half-life increased to 21 days when polyurethane (HYPOL 3000) was used as a matrix. If the immobilized cells were treated with hexamethylene diamine and glutaraldehyde prior

to permeabilization a further increase in stability was found. For agarose-entrapped cells half-life of the isocitrate dehydrogenase activity was 72 days and for the polyurethane-entrapped cells it was 38 days.

However, when glutaraldehyde was used no significant transformation of cathenamine to ajmalicine occurred. Polyurethane entrapped cells showed only 60% activity of agarose-entrapped cells. It should be pointed out that in both these investigations DMSO was present in medium to prevent infections. The most probable reason for the observed low stability is leakage of intracellularly components. The effect of crosslinking reagents and polymers that reacts covalently with the cells can thus be ascribed to a tightening of the cell membrane.

In paper IV a different approach for product release from immobilized plant cells is described. Both plant and animal cells can be stored frozen if they are pretreated and frozen in the presence of DMSO. When they are thawed, removal of DMSO is necessary before they can start to grow. It can thus be concluded that DMSO does not irreversibly alter the cell membrane. As shown in paper IV the permeabilizing effect of DMSO is dependent on several factors, and the optimal procedure has to be determined for each cell line. With cells of *Catharanthus roseus* 5% DMSO is required for complete permeabilization while cells of *Daucus carota* and *Datura innoxia* require 10 and 25% respectively. The reason for these differences may be due to the composition of the cell membrane and/or cell wall or the cell aggregates morphology. The time for permeabilization does not seem to be important since maximal permeabilization occurs within 5 minutes.

The ability of the cells to grow after removal of DMSO is highly dependent on the concentration used for permeabilization. Thus, an increase from 5 to 10% DMSO causes an increase in lag phase from 2 to 8 days for *Catharanthus roseus* cells, figure 8.

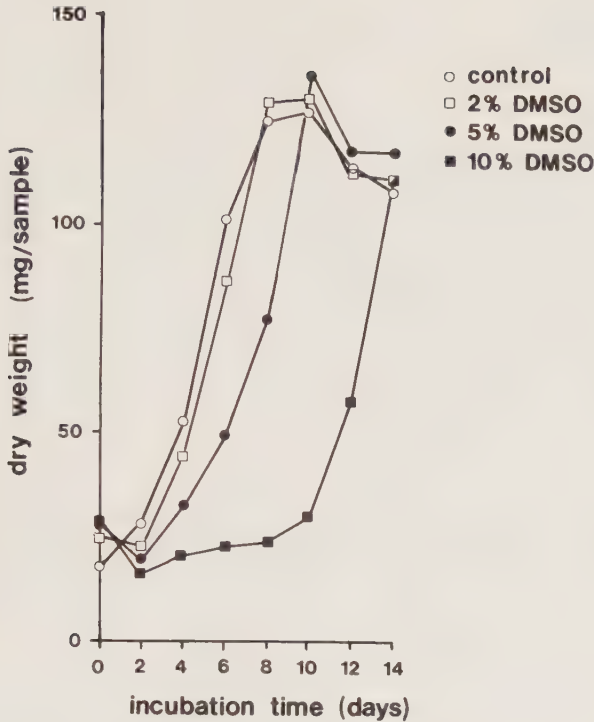


Fig. 8 Dry weight of freely suspended cells of *Catharantus roseus* as a function of incubation time. The cells were treated with the DMSO concentration indicated for 30 minutes before inoculation into growth medium.

Based on this permeabilization method, a general scheme for production of biomedical products from immobilized plant cells can be outlined, figure 9. The immobilized cells are either maintained on a simple medium that promotes production or growth is initiated by addition of complete medium. After a period (which has to be determined empirically) the cells are permeabilized and the product is collected. This procedure can be repeated several times and if the catalytic activity decreases growth can be initiated and thereby increase the operational stability of the biocatalyst. In contrast to the previously described permeabilization method in which the product was continuously released, the product can be harvested by this method in a simple medium, thereby facilitating product purification, and in a high concentration. This method allows reuse of the biomass which is important for a possible commercial utilization of cultured plant cells.

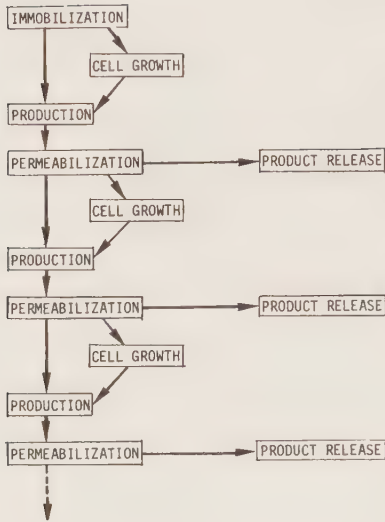


Fig. 9 Schematic diagram of a process for the production of intracellular plant biochemicals using intermittent product release from immobilized plant cells.

In a model experiment according to the procedure outlined in figure 9 the synthesis of ajmalicine from tryptamine and secologanine by immobilized cells of *Catharantus roseus* was studied. Permeabilization was carried out three times and the amount of ajmalicine formed determined. The results are summarized in figure 10. As can be seen the amount of ajmalicine formed increases for each cycle, most likely due to increase in biomass during the growth phase.

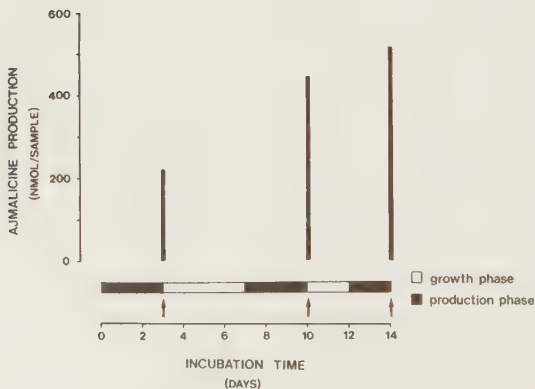


Fig. 10 Ajmalicine production by agarose-entrapped cells of *Catharantus roseus*. The cells were intermittantly permeabilized (arrows).

6. SUMMARY

The thesis is based on 5 papers which can be summarized in the following way:

Animal and plant cells can be entrapped within a polymeric network with retained viability and growth capability. These entrapped cells are able to perform complex biosynthesis and biotransformations. Of particular importance in this context has been the development of a gentle immobilization method. Soy oil or paraffin oil was used as the dispersing medium in the entrapment of cells in beaded polymers. By this way a broad variety of cells have been immobilized with retained viability.

Stored products within immobilized plant cells can be released without affecting the cells viability and thus facilitate development of continuous processes and also makes the reuse of biomass possible.

Microcarriers based on gelatin provides a natural surface for cell growth and makes it possible to release the cells without affecting their viability.

Future development on entrapped animal and plant cells will probably focus on environmental factors such as media requirement, oxygen supply and pH-dependence. These factors have been optimized in free cell systems and will probably be different in an entrapped system.

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PREPARATION OF IMMOBILIZED ANIMAL CELLS

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1. Introduction

In the last years there has been considerable interest in the immobilization of cells particularly those of microbial origin [1,2]. More recently the area has been extended by the reported immobilization of living plant cells in suspension [3].

Here we report on the immobilization of animal cells by adsorption of anchorage-dependent cell cultures to microcarriers such as gelatin beads (including 'magnetic' beads) and chitosan beads. An alternative convenient procedure for harvesting the cells is described. Some results obtained from gel entrapment of various animal cell types will also be given.

2. Materials and methods

2.1. Materials

Chitosan, agarose (type VII), alginic acid (sodium salt, type IV), collagenase (type I, 190 U/mg) were obtained from Sigma. Glutaraldehyde (25%) was from Merck AG. Cytodex and Sepharose CL-6B were products of Pharmacia, Sweden, and the magnetic particles, Fe_3O_4 (5 μm), were a gift from Höganäs, Sweden. Arlacel 83 was obtained from Atlas Chemical Co., dispase (grade II, 0.5 U/mg) was from Boehringer. Gelatin (commercial grade) was from Kebo, Sweden.

2.2. Cell types

The following cells were kindly provided by Professor O. Sjögren: the established cell lines HeLa and K562 (erythroleukemic), and the primary cell cultures S 157N (human skin fibroblasts), S 158A (human kidney carcinoma), DMH W49 (rat colon carcinoma) and DMH W1073 (rat colon carcinoma). All cells except K 562 were grown in Waymouth media supplemented with 20% foetal calf serum. K 562 were grown in RPMI medium with 10% foetal

calf serum. All media were supplemented with gentamicin (50 mg/l).

2.3. Gelatin microcarriers

A gelatin solution (10 ml; 20% (w/v)) obtained by heating to 50°C was dispersed under vigorous stirring in 100 ml of a mixture of toluene:chloroform (73/27, v/v) containing 2% (w/v) of Arlacel 83 at room temperature. After 10 min the mixture was filtered through a 100 μm nylon net whence the collected microcarriers were transferred to acetone. After careful washing with acetone the microcarriers were evaporated to dryness. In order to obtain beads which would resist higher temperatures cross-linking with glutaraldehyde was carried out. Dry beads (1.5 g) were reswollen in 100 ml water followed by the addition of 20 ml 25% glutaraldehyde. After gentle stirring for 30 min the beads were collected and washed with 0.15 M NaCl on a 100 μm nylon net. The beads were dispersed in 0.15 M NaCl and autoclaved at 120°C for 15 min. In order to get a suitable size distribution of the beads they were first filtered through a 250 μm nylon net and the beads in the filtrate were then collected on a 100 μm net. They were then transferred to 0.15 M NaCl and sterilized through autoclaving at 120°C for 15 min. Magnetic microcarriers were obtained by the same procedure except that in addition to 9 ml 20% (w/v) gelatin solution, 1 g Fe_3O_4 particles were added prior to dispersion in the organic phase.

2.4. Chitosan microcarriers

A 2% (w/v) solution of chitosan in 1% formic acid was obtained by mixing chitosan with 1% formic acid and stirring overnight followed by filtration of any undissolved material through a 250 μm nylon net. The chitosan solution (18 ml) was mixed with 2 ml 25% glutaraldehyde and dispersed under vigorous stirring in the same organic phase as described for

gelatin microcarriers. After 15 min the mixture was transferred to methanol. The beads were then washed on a glass filter with methanol and subsequently with 0.15 M NaCl. After autoclaving at 120°C for 15 min (to obtain more rigid beads) the preparation was first filtered through a 250 μ m nylon net after which the beads in the filtrate were collected on a 100 μ m nylon net. The beads were transferred to 0.15 M NaCl and sterilized through autoclaving at 120°C for 15 min.

2.5. Test for cell growth

Before use all microcarriers were washed with 10 vol. medium. Gelatin and chitosan microcarriers (0.25 g wet wt) were mixed with 2.5×10^5 cells (S 157N, S 158A, DMH W49 and DMH W1073, respectively) in 5 ml media in 10 ml siliconized test tubes. They were placed in a CO₂-incubator (5%) at 37°C and mixed in an end-over-end shaker. The cultures were checked daily and the medium was replaced if necessary. The gelatin microcarriers containing Fe₃O₄ were tested with DMH W49. Sepharose CL-6B beads activated with different amounts of CNBr were placed in 20 mm Petri dishes and mixed with 2.5×10^5 DMH W49 cells in 3 ml medium. The cells S 157N and DMH W1073 were allowed to grow on gelatin microcarriers (1 g beads, wet wt) suspended in 50 ml spinners. The starting cell concentrations were 35 000/ml and 22 000/ml, respectively; 60% of the medium volume was replaced each day. For determination of the cell number, duplicate samples of 1 ml suspension were taken. After washing with phosphate-buffered saline (PBS) without Ca²⁺ and Mg²⁺, the beads were incubated with trypsin (0.1% in PBS with 0.02% EDTA and without Ca²⁺ and Mg²⁺) for 15 min at 37°C and the released cells counted in a Bürker chamber.

2.6. An alternative method for harvesting of cells

After 6 days the spinner culture of DMH W1073 was divided into 3 equal fractions after washing as above. They were incubated with 3 ml enzyme solution, trypsin as described above and collagenase (2 mg/ml) in PBS with Ca²⁺ but without Mg²⁺ and dispase (4 mg/ml) in Waymouth medium at room temperature. Samples were taken at intervals and the cell concentration determined.

2.7. Alginate immobilization

Alginate (1 part 4% (w/v)) dissolved in 25 mM Hepes, 125 mM NaCl (pH 7.4) was mixed with 1 part

cell suspension in medium supplemented with twice the concentration of serum. The cell alginate suspension was extruded through a 0.8 mm nozzle into a solution of 25 mM Hepes, 50 mM CaCl₂, 75 mM NaCl (pH 7.4) whereby beads with av. diam. 2 mm were formed. Immobilization was carried out at room temperature. After 5 min the beads were washed with medium and transferred to a spinner flask, which was placed in a CO₂-incubator at 37°C. At intervals beads were withdrawn and dissolved in 0.1 M EDTA (pH 7.4) and the cells were counted.

2.8. Agarose immobilization

Agarose (1 part 4% (w/v)) dissolved in 25 mM Hepes, 125 mM NaCl (pH 7.4) placed in a waterbath at 37°C was mixed with 1 part cell suspension in medium supplemented with twice the concentration of serum. This solution was made into beads by moulding it in a form made of Teflon. Solution was poured over a Teflon plate tightly covered with 3 mm holes. Another plate was used as support and the two were held together by clamps. Before moulding, the form was warmed to 37°C degrees. After the agarose had solidified the form was taken apart and the 'cylindrical beads' were taken out. The beads were put in medium in a spinner flask and placed in a CO₂-incubator at 37°C. At intervals beads were taken out and dissolved by heating to 70°C and the cells were counted.

2.9. Preparation of entrapped β -cells of islets of Langerhans

Ninety isolated islets of Langerhans (av. diam. ~0.1 mm) were prepared from rat [4] pancreas and washed with Krebs-Ringer buffer lacking phosphate [4]. The cells were suspended in 0.125 ml buffer and mixed with 0.5 ml 2.5% (w/v) sodium alginate in the same buffer. The cell alginate suspension was extruded into a solution of the buffer containing 50 mM CaCl₂. After 15 min the beads were washed 3 $\times$ 10 ml with the buffer. Visual inspection showed that the beads contained an average of one islet/bead.

The scintillation vials were each filled with 10 beads and 2 ml buffer, gassed for 1 min with carbogen (95% O₂, 5% CO₂) and preincubated for 30 min at 37°C in a waterbath with shaking. After addition of 1 mg glucose to the vials, they were gassed and 0.1 ml samples taken after 0, 30, 60 and 120 min. The samples were frozen and stored until analyzed for insulin using radioimmunoassay. Visual inspection showed no

release of the islets into the medium.

2.10. Preparation of entrapped adipocytes

Rat adipocytes were prepared according to [5]. For entrapment in alginate the cells were transferred to 20 mM Hepes, 130 mM NaCl, 2% (w/v) albumin (pH 7.4) mixed with an equal volume of 2% (w/v) alginate (autoclaved for 15 min at 120°C) and extruded into a solution of 25 mM Hepes, 50 mM CaCl₂, 75 mM NaCl (pH 7.4). After 5 min the beads were washed with Krebs-Ringer buffer containing 24 mM Hepes, 0.55 mM glucose and 1% (w/v) albumin (storage medium).

With entrapment in agarose the cells were transferred to the above storage medium containing 2% (w/v) albumin and mixed with an equal volume of 6% (w/v) agarose. Beads were prepared as in section 2.8. After gelling the beads were transferred to storage medium.

3. Results

3.1. Cell attachment and growth

In a preliminary test various cells were tested for their capacity to attach to different microcarriers (table 1). All cells could attach to the various beads. Studies on cell growth were subsequently carried out with the most promising microcarrier, gelatin beads with the cells S 157N and DMH W1073. A typical gelatin bead with attached cells is shown in fig.1. As seen from fig.2 no indication of cell death occurred and good growth was observed.

3.2. Harvesting of cells

In fig.3 the results from three different enzymic procedures leading to cell detachment from gelatin microcarriers are given. As seen, both collagenase and

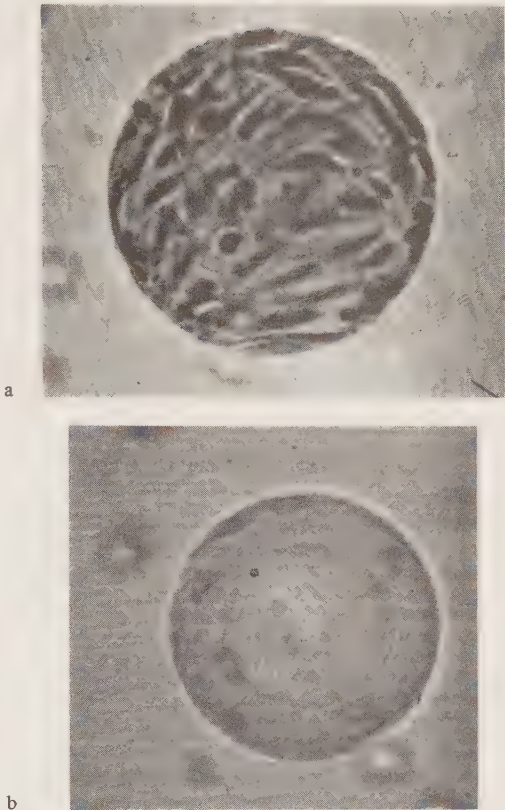


Fig.1. Photographs show: (a) a representative gelatin bead taken from a culture of DMH W49 after 48 h; (b) a gelatin bead prior to inoculation.

dispase give rise to a far higher total cell concentration than normally applied trypsin treatment.

3.3. Cell entrapment

I. Isolated cells: In a preliminary study to test whether animal cells would survive entrapment in

Table 1
Attachment of primary cell cultures to different microcarriers

	S 157N	S 158A	DMH W49	DMH W1073
Gelatin	+	+	+	+
Gelatin (magnetic)	n.t.	n.t.	+	n.t.
Chitosan	+	+	+	+
CNBr-activated Sepharose CL-6B	n.t.	n.t.	+	n.t.

n.t., not tested

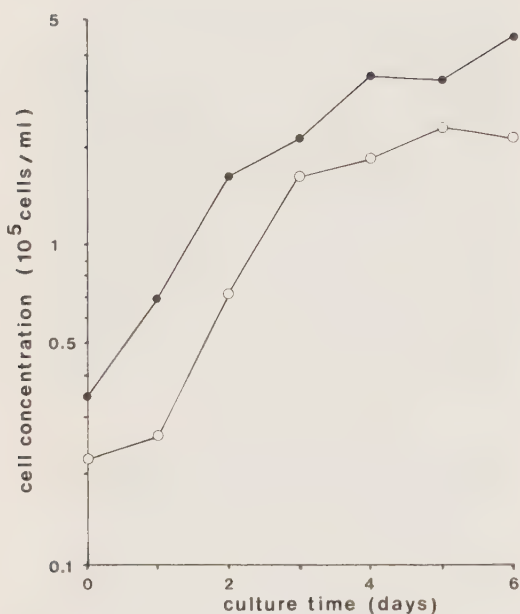


Fig. 2. The growth of two primary cell cultures, S 157N (●—●) and DMH W1073 (○—○), on gelatin beads in spinner. The figures given represent the mean of 2 cell counts.

a gel matrix hepatocytes from rats were isolated [6] and entrapped in calcium alginate. The obtained beads were subsequently incubated in perfusion buffer and water, respectively. Release of lactate dehydrogenase was monitored. About 10-times lower leakage of the enzyme was found in the sample kept in the perfusion buffer compared to the sample in water. Subsequent incubation in water of the beads previously kept in perfusion buffer however led to considerable leakage of active lactate dehydrogenase. Subsequently islets of Langerhans containing β -cells were isolated and entrapped following the same procedure to check whether the latter were capable of insulin production/secretion in the immobilized state. Over 0, 30, 60 and 120 min samples were withdrawn from the medium and assayed for insulin content using radioimmunoassay analysis. The amount of insulin secreted was 1, 23, 40 and 78 immunoreactive insulin units (μ U/ml), respectively.

Finally adipocytes were isolated and entrapped in both Ca-alginate as well as agarose. They were subsequently tested for their capability to incorporate [3 H]glucose into lipids [7] following insulin

stimulation as well as for their ability to release free fatty acids by noradrenaline stimulation [8]. It was found that, at the outset, the immobilized adipocyte preparation showed a higher basal incorporation than free cells which was more pronounced with cells immobilized in Ca^{2+} -alginate. On stimulation with insulin both preparations showed an increase in glucose incorporation (when higher concentrations of alginate were used, the cells did not respond to insulin in the glucose test). The free fatty acid release was only studied with agarose immobilized adipocytes as the alginate beads were too soft. The immobilized cells also showed a response to added noradrenaline by releasing free fatty acids after about twice the time required for free cells, and at $\sim 1/3$ rd the rate for free cells.

II. Cell cultures: A number of cell types, i.e., fibroblasts, HeLa, DMH W49 and K 562, the latter growing in suspension, were entrapped in gels to test whether they would grow within a gel-matrix or remain viable. The gels prepared were beads of: (a) 2% alginate; (b) 2% agarose; (c) a mixture of 2% agarose and 0.5% alginate; and (d) 2% agarose containing simultaneously entrapped Cytodex particles to which cells already had been attached. In all cases serum was entrapped simultaneously with the cells as in-diffusion of some of the components of serum was likely to be hindered. No proliferation of the cells tested was observed

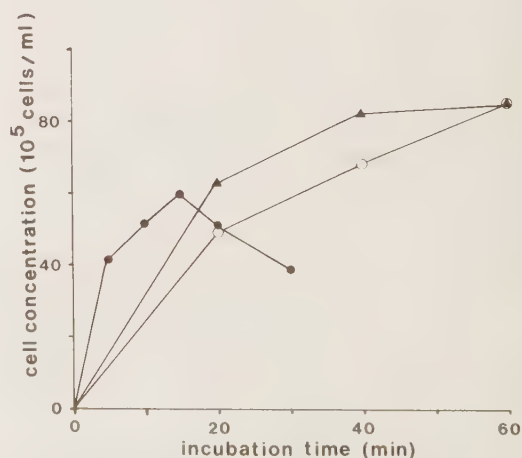


Fig. 3. Release of microcarrier-bound cells using trypsin (●—●), collagenase (○—○) and dispase (▲—▲), respectively.

under the conditions used; however on average, 10–30% of the cells remained viable as judged by the trypan blue exclusion test, after 1 week of incubation. In controls run with HeLa cells non-entrapped but kept free in solution in siliconized tubes they had disappeared after 1 day.

4. Discussion

The use of microcarriers for culture of mammalian anchorage-dependent cells in suspension has been given increasing attention in the last years. Following the original experiments using DEAE–Sephadex [9], microcarrier beads are now commercially available based on related supports as well as those based on polyacrylamide carrying positively charged dimethylaminopropyl groups. Although the above carriers have proven most valuable there seemed room for improvement to obtain cheaper supports and from which cells, if required, could be removed more easily.

In the course of our studies to obtain a suitable gel matrix for entrapment of animal cells we found, in analogy to [10], that collagen substrate adhered to cells. On further investigation a simple technique leading to the preparation of solid beads, 100–250 μm , of the closely related gelatin (the product obtained on boiling collagen) was developed. All the cells tested attached and proliferated. In this context 'magnetic' gelatin beads were also prepared and as expected no adverse effect on cell growth was observed. Potentially, such supports would permit facile recovery when used in media of viscous or particulate nature. Furthermore, it is conceivable to suspend the beads by applying an outer magnetic field thereby minimizing the need for vigorous agitation when using carriers of high specific weight.

Beads of the ubiquitous chitosan, a partially deacetylated product of the polysaccharide chitin, were also prepared using a similar procedure and found to lead to attachment of cells. Their properties as supports for cell growth will be tested further. In our investigations to find supports for cell growth of anchorage-dependent cells we coupled polylysine covalently to CNBr-activated agarose beads. Although nothing definite can be said at this point on the attachment capacity of such preparations for the cell types tested it was found that agarose alone activated with high concentrations of CNBr did lead to attachment and

growth of cells, probably due to the positive charge introduced on the matrix.

In our experiments involving detachment of cells we attempted an alternative approach as there was the possibility with the new microcarriers used to enzymically dissolve the support directly. Both dispase and collagenase dissolved the beads leading in the case of dispase to a clear solution. In contrast to trypsin treatment which involves destruction of the cell surface to accomplish cell removal the approach taken here should leave the entire cells intact and viable which is an advantage, e.g., when the cells are used for immunization or when analysis of surface antigens will be done. Summarizing, as gelatin is inexpensive and represents a more 'natural' surface for cell attachment than those commercially available, it appears that such microcarriers will turn out to be valuable supports for cell growth as also strongly indicated by the spinner culture experiments reported here. The ease of cell release adds to the merits of such microcarriers.

The studies reported here on the entrapment of animal cells in gels (detailed in [11]) were initiated because such immobilized preparations should offer the same advantages as those observed with micro-organisms or plant cells. For instance, isolated cells when immobilized in the entrapped state would allow convenient assay in analytical metabolic perfusion studies or could be used for the enzymic conversion of certain metabolites by analogy to steroid transformation using entrapped bacteria [12] or be utilized for, e.g., hormone production. These data demonstrate that cell entrapment leaves at least the major part, if not all, of their metabolic machinery intact. We applied particularly one of the more recently developed entrapping techniques, i.e., inclusion in calcium alginate [13–15].

It could be demonstrated with isolated rat hepatocytes that the cells remained, at least partially, intact on immobilization, as disruption of the cell membrane would have lead to leakage of the enzyme, lactate dehydrogenase. Subsequently islets of Langerhans from mouse pancreas were immobilized by the same procedure giving preparations still capable of insulin production/secretion [16]. We also showed that adipocytes, entrapped in either calcium alginate or agarose, were still capable of metabolizing added radioactive glucose to fatty acids and could be stimulated further on addition of insulin [17].

A drawback of the calcium alginate procedure is

the requirement for Ca^{2+} or similar ions to keep the network intact and following this the necessity to operate in phosphate-free media. Entrapment in agarose particles, as described here and applied in the studies of free fatty acid release of adipocytes, may offer an alternative approach.

With regards to anchorage-dependent cells, good microcarriers including those described here are at hand, whereas for suspension cultures entrapment appears the best alternative for immobilization. Furthermore, anchorage-dependent cells might profit from entrapment within a three-dimensional network while adsorbed to their normal carrier as this would give some protection against, e.g., shear force. It may even be that anchorage-dependent cells for which no suitable microcarrier has yet been found may accept the polymer network as recipient attachment surface or at least remain viable as demonstrated here using trypan dye exclusion tests. Although no clear cell proliferation has yet been observed with the various entrapped cell types they remain viable for a considerable time and permit metabolic studies, enzymic transformations or syntheses to be carried out.

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ENTRAPMENT OF PLANT CELLS IN DIFFERENT MATRICES

A comparative study

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1. Introduction

During recent years the interest for plant tissue cultures as producers of secondary metabolites of commercial value has increased considerably [1–3]. Both callus and suspension cultures might be used in principle, but the latter type is preferred. Low productivity of natural products in such tissue cultures is, however, a recognized problem and many attempts are being made to manipulate the overall metabolism of the cells in order to increase the total yield of the product in question.

We reported the immobilization of whole plant cells by entrapment in calcium alginate gels [4] and believe that this technique is a valuable addition to the general techniques used in plant tissue cultures. The production of secondary products by the immobilized cells was higher than that for freely suspended cells under the same conditions. Thus, immobilized cells of *Morinda citrifolia* produced up to 10-times as much anthraquinones as freely suspended cells [5].

Immobilized plant cells were later studied for bio-transformations of natural products [6–8]. The immobilization technique used in [6–8] was also entrapment in calcium alginate gels. Such gels require Ca^{2+} as stabilizer, which can lead to problems in phosphate-containing media. Therefore in [7] a special medium lacking phosphate ions was designed.

In our initial studies on immobilized plant cells we chose to use entrapment in alginate because there are also distinct advantages with this immobilization technique, including reversibility of the immobilization which enables the investigation of the cells in free suspension subsequent to their confinement in the immobilized state.

Here, we describe studies on alternative methods

for the immobilization of plant cells. All experiments have been carried out with cells of *Catharanthus roseus*, obtained from suspension cultures. After immobilization by entrapment in various gels the viability of the gels (tested by plasmolysis, respiration and cell growth) as well as the ability of the immobilized cells to synthesize an indole alkaloid (ajmalicine) both from precursors (tryptamine and secologanin) and de novo with sucrose as sole carbon source has been investigated.

2. Materials and methods

2.1. Materials

Agar was supplied by Difco Labs. (Detroit, MI) and agarose (type VII) and κ -carrageenan (type III) by Sigma (St Louis, MO). Alginate (Manucol DH, batch no. 430041 E 6896) was obtained from Alginate Industries (Girven) and gelatin (commercial grade) from Kebo (Sweden). [$2\text{-}^{14}\text{C}$]Tryptamine was from NEN (Boston, MA) and secologanin and ajmalicine were kind gifts from Dr M. H. Zenk. All other chemicals and biochemicals were purchased from commercial sources.

2.2. Cultivation of *Catharanthus roseus*

The cell suspensions of *Catharanthus roseus* were cultivated in the medium of [9] supplemented with 10^{-6} M 2,4-dichlorophenoxyacetic acid and 10^{-6} M naphthylacetic acid (pH 6.0) on a rotary shaker at 100 rev./min and at 26°C.

2.3. Immobilization of *C. roseus* cells

The immobilization of *C. roseus* cells in various gels was carried out under sterile conditions in the following way:

Entrapment in calcium alginate was done as in [4] by the method in [10]. The cells (5.0 g wet wt) were suspended in 5% alginate (5.0 g) and the suspension was added dropwise to 50 mM CaCl_2 . The alginate beads formed were left for 1 h in this solution and then washed.

Immobilization in a copolymer of alginate and gelatin was carried out in a similar way. The cells (5.0 g wet wt) were suspended in a mixture of 5% alginate (3.75 g) and 20% gelatin (1.25 g) and beads were made as above. The beads were then treated with 2% glutaraldehyde (10 ml) for 30 min and then washed again.

Agarose-entrapped cells were prepared by the method in [11]. The cells (5.0 g wet wt) were suspended in 5% agarose (5.0 g) at 40°C. As quickly as possible this suspension was poured over a Teflon plate tightly covered with holes (diam. 3 mm). Another plate was used as support and the two plates were held together by clamps. After the agarose had solidified the form was taken apart and the 'cylindrical beads' were taken out and washed.

Cells were entrapped in a copolymer of agarose and gelatin in a similar manner. The cells (5.0 g wet wt) were suspended in a mixture of 5% agarose (3.75 g) and 20% gelatin (1.25 g) and cylindrical beads were made as above. The washed beads were treated with 2% glutaraldehyde (10 ml) for 30 min and then washed again.

Gelatin-entrapped cells were obtained as follows: The cells (5.0 g wet wt) were suspended in 20% gelatin (5.0 g) and 25% glutaraldehyde (0.4 g) was added. Cylindrical beads were moulded as above and after 30 min the beads were taken out and washed.

Cells were immobilized in carrageenan as follows: The cells (5.0 g wet wt) were suspended in 3% carrageenan (5.0 g) at 50°C and the suspension was as soon as possible added dropwise to 0.3 M KCl. The carrageenan beads formed were left for 1 h in this solution, then washed.

Cells were immobilized in agar by suspending them (5.0 g wet wt) in 4% agar (5.0 g) at 50°C with subsequent moulding of cylindrical beads.

Finally, cells were entrapped in polyacrylamide as follows: The cells (5.0 g wet wt) were suspended in a solution of acrylamide (1.45 g) and *N,N'*-methylendi-acrylamide (0.07 g) in 0.05 M Tris-HCl buffer (pH 7.5) (5.0 ml). To this suspension was added TEMED (0.02 g in 0.2 ml H_2O) and ammoniumpersulfate (0.02 g in 0.4 ml H_2O) and beads were moulded in the Teflon-device.

2.4. Cell growth of immobilized *C. roseus*

Cultivation of immobilized *C. roseus* cells in the medium of [9], supplemented with hormones, was initiated by inoculating beads (1.0 g) of the various preparations in samples of the medium (10 ml). The flasks were shaken at 100 rev./min and at 26°C. Cell growth was estimated by observing the number of freely suspended cells in the medium.

2.5. Biosynthetic studies

The synthesis of ajmalicine from tryptamine and secologanin by free and immobilized cells was studied in the following way: Free cells (0.5 g wet wt) or immobilized cells (1.0 g beads) were added to medium [9] (10 ml) with no hormones added, containing tryptamine (1.25 μmol) labelled with ^{14}C (400 000 dpm/ μmol) and secologanin (1.25 μmol). After 2 and 5 days samples were analyzed for radioactive ajmalicine.

The de novo synthesis of ajmalicine was determined in a similar manner. Thus, free cells (0.5 g wet wt) or immobilized cells (1.0 g beads) were inoculated in the hormone-free medium (10 ml). After 2 weeks samples were taken and analyzed for ajmalicine content.

2.6. Analytical procedures

Plasmolysis of cells with glycerol was followed under microscope with phenosafranin as indicator. Before the immobilized cells were studied the beads were cut into thin slices.

Respiration of the cells was monitored with an oxygen electrode (Rank Brothers). Free cells (0.1 g wet wt) or immobilized cells (0.2 g beads, chopped into small pieces) were added to the growth medium (2.0 ml) and the consumption of oxygen was followed on a recorder.

Ajmalicine extracted as follows: The medium was extracted with methylene chloride (3 × 5 ml). The organic phases were combined and evaporated to dryness under vacuum on a rotavapor. The residue was dissolved in either chloroform (1.0 ml) for the experiments with precursors or in the HPLC-eluent (1.0 ml) for the de novo experiments.

The cells/beads were placed in a Soxhlet-extractor and methanol (25 ml) was used for the extraction which was done overnight in a water bath. The methanol was evaporated and the residue was dissolved in either chloroform or HPLC-medium as above.

The amount of radiolabelled ajmalicine was esti-

mated after TLC-chromatography on silica plates (DC-Alufolien, Kieselgel 60 F254, E, Merck) with acetone–petroleum ether–diethylamine (2:7:1) as eluent. Samples (50 µl) from the extractions were applied and ‘cold’ ajmalicine was added to each sample in order to simplify detection of the ajmalicine-spots (R_F 0.58), which were cut out and analyzed for radioactivity.

The samples from the de novo synthesis experiments were analyzed by HPLC under the following conditions:

Column size:	200 × 4 mm
Column packing:	Nucleosile- C_{18} , 5 µm
Eluent:	0.01 M (NH ₄) ₂ CO ₂ –acetonitril (3:7)
Flow rate:	1.0 ml/min
Sample size:	10 µl
Detection:	UV, 242 nm

The amount of ajmalicine (retention time 5.4 min) was determined by measuring the peak heights and comparing them to standards. The detection limit is ~0.5 µg/ajmalicine/ml.

3. Results and discussion

Cells from suspension cultures of *Catharanthus roseus* were immobilized by entrapment in various gels (see table 1). Some characteristics of the immobilized plant cells were studied and a comparison of the different immobilization methods was done as described below.

3.1. The plasma membrane

An intact plasma membrane is of great importance when a retained viability of the cells is desired after the immobilization. Plasmolysis was used as an indica-

tion of the intactness of the plasma membrane of the cells. The immobilized cells were thus exposed to a plasmolysing agent, e.g., glycerol, and studied under microscope. The dye phenosafranin, which does not penetrate an intact membrane, was used as an additional indicator. Furthermore, such a dye simplifies the detection of plasmolysis.

As can be seen in table 1 all the immobilized cells except for those entrapped in polyacrylamide were plasmolysed and thus appeared to have intact plasma membranes. Obviously, the chemicals used in the polyacrylamide entrapment are harmful to the cell membrane. The plasmolysis experiments were carried out directly after the immobilization as well as after 20 h. No significant difference was observed between these two sets of experiments, indicating a relatively good stability of the immobilized cells. For the fully viable cells (see under cell growth below) the plasma membrane appeared to stay intact over an extended period of time (up to 2 weeks; longer incubation times not tested).

3.2. Respiration

A second indication of viability is retained respiration of the cells after immobilization. Respiration of the cells was measured with an oxygen electrode. To reduce diffusion barriers the beads containing the plant cells were chopped into relatively small pieces before the measurements. As can be seen in table 1 the cells within the gels treated with glutaraldehyde (i.e., the gels containing gelatin) lost their respiration capacity. The respiratory chain of these cells appears to be sensitive to glutaraldehyde. It may, however, be possible to crosslink with glutaraldehyde under milder conditions [12], which may leave the respiratory chain intact. Furthermore, it can be pointed out that this study on respiration is only qualitative since some diffusion barriers still exist after the beads have been chopped into pieces.

The cross-linking with glutaraldehyde was attempted in order to stabilize the gels. In the case of alginate such a stabilization by cross-linking could obviate the requirement for high calcium concentrations in the medium and thereby eliminate problems with the administration of phosphate to the immobilized plant cells.

3.2. Cell growth

The ultimate criterion on viability is, of course, the growth and division of cells. As can be seen in

Table 1
Comparison of various preparations of immobilized *C. roseus* cells

Preparation	Plasmolysis	Respiration	Cell growth
Alginate	+	+	+
Agarose	+	+	+
Agar	+	+	+
Carrageenan	+	+	+
Alginate + gelatin	+	–	–
Agarose + gelatin	+	–	–
Gelatin	+	–	–
Polyacrylamide	–	–	–

table 1 the cells entrapped in polyacrylamide and in the gels treated with glutaraldehyde did not, as expected, grow at all and therefore these preparations were not tested further. On the other hand, when the cells immobilized in agar, agarose or alginate were placed in a complete medium they grew and divided and after 1 week the number of cells had increased to such an extent that the beads started to burst. After a few additional days most of the cells were found in a free suspended form. On a semi-quantitative basis it was concluded that the cells entrapped in agar and agarose grew better than the cells immobilized in alginate. The cells immobilized in carrageenan did not grow to such an extent but after 1 week a number of cells could be found in the medium. Obviously, the elevated temperatures (up to 50°C) required for the entrapment in agar, agarose and carrageenan did not influence the viability of the cells to any great extent.

In the biosynthetic studies (see below) the immobilized cells were placed in a growth limiting medium, i.e., no hormones were added. The number of cells did not increase to any great extent in such a medium and after 2 weeks incubation no free cells were observed in the medium.

3.4. Synthesis of ajmalicine from precursors

Cells of *C. roseus* entrapped in alginate can synthesize ajmalicine from the distant precursors tryptamine and secologanin [4]. The various preparations of immobilized cells were incubated with these precursors and the production of ajmalicine was determined. The results are given in table 2 and it can be seen that relatively high incorporations of [¹⁴C]tryptamine were observed for all the preparations studied. The increase in incorporation between day 2 and day 5 was relatively small but relative to freely suspended cells the increase for the immobilized cells was larger. Further-

more, it can be concluded that the cells entrapped in agarose or alginate were more efficient than free cells under the conditions used. Cells entrapped in agar and carrageenan were on the other hand somewhat less effective.

3.5. De novo synthesis of ajmalicine

We have also studied the de novo synthesis of ajmalicine with sucrose as the sole carbon source by immobilized cells of *C. roseus*. As can be seen in table 2 all the preparations of immobilized cells were capable of synthesizing ajmalicine at a rate 60–140% of that observed with freely suspended cells. There are several reports on the de novo synthesis of ajmalicine by *C. roseus* cells in suspension cultures [13–15]. Obviously, the ability to synthesize ajmalicine is preserved in the immobilized cells and even enhanced in the case of alginate entrapped cells. The synthesis of ajmalicine can be increased considerably for both free and immobilized cells by using the alkaloid production medium in [13]. The reason for the increased synthesis observed with alginate entrapped cells is at present under investigation and will be reported elsewhere [16].

4. Conclusions

These data clearly demonstrate that plant cells can be immobilized by various methods with preserved metabolism. The secondary product formation (from precursors or de novo) is in the cases studied highest for alginate entrapped cells but also agar, agarose and carrageenan appear to be suitable polymers for entrapment of plant cells. Furthermore, it can be concluded that plant cells are sensitive to reactive chemicals (e.g., glutaraldehyde) which are often used for the immo-

Table 2
Synthesis of ajmalicine by free and immobilized cells of *C. roseus*

Cell prepn.	From precursors				De novo	
	2 days		5 days		2 weeks	
	% Incorp.	Rel. incorp.	% Incorp.	Rel. incorp.	µg/sample	Rel. prod.
Free cells	7.2	100	7.9	100	4.2	100
Agar	6.4	89	7.5	95	3.7	88
Agarose	7.8	108	9.0	114	4.2	100
Alginate	9.1	126	13.9	176	5.9	140
Carrageenan	5.5	76	6.5	82	2.6	62

bilization of biocatalysts to various supports. Consequently, entrapment seems to be the most suitable method for immobilization of plant cells.

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A GENERAL METHOD FOR THE IMMOBILIZATION OF CELLS
WITH PRESERVED VIABILITY

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Running title: Immobilization of viable cells

SUMMARY

Microbial, algal, plant and animal cells have been immobilized, with preserved viability, by entrapment in various matrices according to a new bead polymerization technique. The cell polymer/monomer mixture is kept suspended in a hydrophobic phase such as soy, paraffin, or silicon oil, tri-n-butylphosphate, or dibutylphthalate, which is compatible with the cells. The various monomers or polymers tested include agarose, agar, carrageenan, alginate, fibrin, and polyacrylamide. Furthermore, by adjustment of the stirring speed of the suspension, beads of desired diameter can easily be obtained. The entrapped cells are fully viable and biosynthetically active.

1. INTRODUCTION

Immobilized whole cells entrapped in polymeric matrices have, during recent years, been studied extensively for biotechnological applications (Cheetham 1980, Birnbaum *et al.* 1982). In most instances a single enzymic activity present in non-viable cells has been utilized. The potential usefulness of immobilized cells in order to carry out complex biochemical conversions and syntheses is great, however usually requires intact and viable cells. The lack of suitable techniques with which to immobilize whole cells while preserving their viability has perhaps to some extent, hampered their use.

Optimal immobilized catalyst preparations usually require spherical particulates as they are homogeneous and facilitate column packing. In the past spherical particulates of some polymers containing entrapped enzymes have been made by carrying out gel formation in an organic phase such as toluene:chloroform (Nilsson *et al.* 1972), which, however, does not leave entrapped cells in a viable state. In this communication we wish to report on a newly developed mild immobilization technique resulting in a beaded uniform catalyst. The method (schematically outlined in Fig. 1), which can be used for a wide variety of polymers, is based on a two-phase system in which the hydrophobic phase is composed of an inert liquid such as vegetable oil or tri-n-butylphosphate. We have immobilized microbial, algal, plant and animal cells while retaining their viability and biosynthetic capacity using this general procedure.

2. MATERIALS AND METHODS

2.1. Materials

Agar and nutrients were supplied by Difco Labs. (Detroit, MI.) and κ -carrageenan by Tanabe Seiyaku Co., Ltd. (Osaka, Japan). Agarose (type VII), fibrinogen (type 1-S) and β -sitosterol were obtained from Sigma Chemical Co. (St. Louis, MO.). Alginate (Manucol DH) was from Alginate Industries (Girven, UK) and trombin (topostatin) was from F. Hoffman-La Roche & Co. AG (Basel, Switzerland). GAFAC (RS-410) was purchased from GAF Corporation (New York, N.Y.) and Arlcel 83 from Atlas Chemie (Essen, FRG).

2.2. Cultivation of organisms

Mycobacterium sp. (NRRL B-3683) was cultivated in a medium containing 0.8 % nutrient broth, 0.1 % yeast extract and 0.1 % myo-inositol, pH 7.0, for 2 days at 28-30 °C.

Gluconobacter oxydans subsp. *melanogenus* (ATCC 15163) was cultivated at pH 7.0 in a medium containing 2 % sorbose, 0.5 % glycerol, 0.5 % yeast extract, 0.5 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.5 % CaCO_3 , for 2 days at 28-30 °C.

Pseudomonas putida (ATCC 21812) was cultivated in a medium containing 0.8 % nutrient broth, 0.4 % sorbose, 0.5 % glycerol and 0.5 % CaCO_3 , pH 7.0, for 2 days at 28-30 °C.

Glycine max (soy bean) was cultivated in B5-medium as described by Gamborg *et al.*, 1968, supplemented with 1 ppm 2,4-dichlorophenoxyacetic acid.

The erythroleukemic cell line K-562 was grown as described previously (Nilsson and Mosbach 1980).

Bakers yeast (*Saccharomyces cerevisiae*) was obtained locally from a commercial supplier.

2.3. Immobilization of cells

Cells were immobilized by entrapment in various matrices, as summarized in Table 1, according to the following standard procedures (Nilsson and Mosbach 1982). When required the whole procedure was carried out under sterile conditions.

A. Agar and agarose

A warm aqueous solution of the polymer (8 g) is mixed with the cells (2 g wet weight) and the suspension is subsequently dispersed in the oil phase (40 ml) by magnetic stirring. When droplets of appropriate size have been formed the mixture is cooled on an ice-bath under continued stirring until the polymer has solidified. The mixture is then transferred to centrifuge tubes, after which water, buffer or medium is added. The beads are spun down (2 min; 100 x g), the upper oil phase and most of the aqueous phase removed with an aspirator and the washing procedure repeated if necessary.

B. Carrageenan

The polymer is dissolved in warm 0.9 % NaCl. The preparation of beads is carried out as described above. The beads are, however, washed with 0.3 M KCl for improved stability.

C. Polyacrylamide

A stock solution of monomers is made up from acrylamide (17.6 g) and N,N'-methylene-bis-acrylamide (1.2 g) dissolved in 100 ml of 0.05 M Tris-HCl buffer, pH 7.0. Monomer solution (8 ml) is mixed with cells (2 g wet weight) ammonium persulfate is added (20 μ l; 0.4 g/ml) and the suspension is dispersed in an oil phase (40 ml) supplemented with 0.05 % Triton X-100. Tetramethylenediamine (100 μ l) is added when droplets of appropriate size have been formed. After polymerization the beads are collected and washed as described above.

D. Fibrinogen

A fibrinogen solution (2 % w/v; 3 ml) is mixed with the cell suspension (3 ml) and thrombin (15 U) is added. The suspension is poured into paraffin oil (60 ml) which has been extracted with phosphate buffered saline, pH 7.0, and supplemented with 0.05 % Triton X-100. After 5 min growth medium (100 ml) is added. The oil is removed after the beads have sedimented. The beads are then washed with medium and cultivated.

E. Alginate

Sodium alginate (5 % w/v; 10 ml) was mixed with cells (1 g wet weight) and this mixture was subsequently added to an oil phase (50 ml) which had been supplemented with 0.1 % GAFAC RS-410. The resulting suspension was stirred until small droplets had formed and then it was rapidly mixed with 0.05 M CaCl_2 (300 ml). The alginate beads were allowed to stabilize for 15 min and then the beads were collected on a nylon net (100 μ m) and

the oil was washed away with 0.05 M CaCl_2 .

2.4. Analytical procedures

Cellular respiration was measured by monitoring the oxygen consumption with an oxygen electrode (Rank Brothers, UK). The system contained 0.1 g of free cells (wet weight) or the corresponding amount of immobilized cells suspended in growth medium to a final volume of 2.0 ml.

Growth of soy bean cells in suspension cultures was measured by determining their dry weight as a function of incubation time. Samples were collected on preweighed filter papers and dried at 60 °C until a constant weight was reached.

Ethanol, steroids and 2-ketogulonic acid were analyzed by HPLC according to the conditions listed in Table 2. Steroids were extracted from the incubation mixture with heptane before analysis.

Yeast cells were stained by immersing the beads in 1 % Fuchsin for 5 min followed by extensive washing with water. K-562 cells were stained for 5 min in Türk's solution.

3. RESULTS AND DISCUSSION

Immobilized viable cells are likely to find increasing biotechnological applications as such preparations can be advantageously utilized for the synthesis and transformation of highly complex compounds. Studies in this direction include the formation of hormones and antibodies by immobilized animal cells (Nilsson *et al.* 1982) and of alkaloids by immobilized plant cells (Brodelius *et al.* 1979, Brodelius and Mosbach 1982). Probably the most widely used technique for the immobilization of cells with preserved viability to date has been their entrapment in Ca^{2+} -alginate or κ -carrageenan. One of the major advantages of this technique is the simplicity with which spherical particals can be obtained simply by dripping a polymer-cell suspension into a medium containing positively charged ions. There are, however, a number of other polymers with desirable properties which also should be useful for the immobilization of viable cells. These polymers would have the advantage over alginate in that they would not require relatively high concentrations of counterions for gel formation. However, the difficulties involved in the preparation of spherical particales with these polymers

have significantly hampered their wider use.

We wish to report here on a convenient and generally applicable method of cell entrapment which leads to spherical particles and in which the cells remain viable. The main feature of the process is the use of oil as a suspension medium. From the literature we know only of one report in which cells containing penicillin amidase were entrapped in polyurethane by using an oil phase (Klein and Kluge 1981). No mention, however, was made as to whether or not the cells remained viable within this polymer or were capable of carrying out complex enzymic reactions. We wish to present data on the use of the technique in the entrapment of microorganisms, algal, plant and animal cells leading to preparations with retained viability.

The technique is based on a two-phase system consisting of an aqueous and an inert hydrophobic phase as outlined in Fig. 1. The cells to be immobilized are mixed with a polymer/monomer solution and subsequently this cell-polymer/monomer suspension is poured into the hydrophobic phase under stirring. When droplets have been formed the polymer is induced to solidify (for instance by cooling or cross-linking) or, in the case of e.g. acrylamide monomers, allowed to polymerise. The size of the beads can easily be adjusted by the stirring speed. Relatively small beads ($\phi = 50-100 \mu\text{m}$), containing immobilized cells are readily prepared. The size distribution of these beads can, to some extent, be controlled. If necessary the beads can be sieved on metal screens. When a melted polymer (e.g. agarose, agar or carrageenan) is used for the immobilization, the temperature of the polymer solution is adjusted to 5-10 °C above the gelling temperature. The cells are added and subsequently the suspension is quickly poured into the hydrophobic phase which is also kept at the same elevated temperature. Cooling is carried out under stirring by transferring the mixture to an ice bath. It should be pointed out that the entire immobilization procedure is quite easily performed aseptically.

The influence of the hydrophobic phase on the viability of the entrapped cells has been investigated. Plant cells (soy bean) grown in suspension cultures were selected for these studies since such cells are in general rather sensitive to changes in the environment, particularly in comparison with microbial cells. A convenient measure of the viability was obtained by monitoring the respiration of the immo-

bilized cells with an oxygen electrode. The results are summarized in Table 3. A relatively high respiration rate was observed for all preparations of agar-entrapped soybean cells (*Glycine max*) except for the one prepared in a toluene:chloroform mixture. Yeast cells entrapped in polyacrylamide showed the same behavior (Table 3). Toluene:chloroform mixtures have been used earlier in our laboratory for the successful immobilization of various enzymes in polyacrylamide beads (Nilsson *et al.* 1972). The organic solvents are, however, too aggressive for immobilising living cells. Most likely various membranes within the cells are affected by the solvents. As seen from Table 3 a relatively broad spectrum of compounds can be employed as the hydrophobic phase in the two-phase system described here for the entrapment of sensitive cells.

Respiration of soybean cells entrapped in various matrices (prepared in soy oil) over an extended period of time is shown in Fig. 2. Fig. 3 illustrates the cell growth (dry weight increase) for the corresponding preparations. While the cells entrapped in agar or agarose respire and grow at almost the same rate as freely suspended cells, the cells entrapped in alginate (prepared as described by Brodelius and Nilsson 1980) are somewhat restricted in respiration and growth, as has been reported previously (Brodelius *et al.* 1982). Obviously, the viability of the entrapped cells is not changed to any great extent during the immobilization in the two-phase system.

As well, the biosynthetic capacity of plant cells is retained after immobilization according to the present method. Cells of *Daucus carota* entrapped in agar, agarose or carrageenan can efficiently 5- β -hydroxylate digitoxigenin to periplogenin (Linse and Brodelius, unpublished) and cells of *Catharanthus roseus* entrapped in agarose can synthesize the indole alkaloid ajmalicine from the distant precursors tryptamine and secologanin (Brodelius and Nilsson 1982).

Yeast cells (*Saccharomyces cerevisiae*) were entrapped in agarose and carrageenan with soy oil as hydrophobic phase. The fermentation capacity of these preparations was compared to that of cells entrapped in Ca^{2+} -alginate according to previous studies (Larsson and Mosbach 1979). Fig. 4 shows the fermentation capacities of these preparations. Comparable fermentation capacities were observed in all cases.

To demonstrate that the yeast cells are viable, the preparations were activated *in situ* by placing the beads in a nutrient medium as previously

described (Larsson and Mosbach 1979). The fermentation capacities of the three preparations tested increased 80-160 fold by this activation procedure (summarized in Table 4). We ascribe the increased fermentation capacity at least partly to the increased cell number within the beads. A more direct evidence for cell growth is shown in Fig. 5. Yeast cells were immobilized in agarose (paraffin oil at 40 °C) at a very low cell density (10-20 cells/bead) and subsequently the beads were placed in nutrient medium for 12 h. As can be seen in Fig. 5B, small cell colonies have been formed within the polymer matrix during the incubation. With the present method, yeast cells can be immobilized in various matrices with preserved viability and fermentation capacity.

Algal cells (*Chlorella vulgaris*), entrapped in agarose beads (prepared in soy oil at 40 °C), are photosynthetically active, as has been reported elsewhere (Wikström *et al.* 1982). We have for some time been involved in the development of a process for the production of α -keto acids based on immobilized microorganisms containing an amino acid oxidase (Brodelius *et al.* 1981, Szwajcer *et al.* 1982). The conversion of amino acids to the corresponding keto acids with such preparations is, however, highly dependent on the oxygen transfer step. When algal cells are co-immobilized with the bacteria, an internal production of oxygen is possible. A tenfold increase in productivity was observed when co-immobilized cells were illuminated in a specially designed reactor (Wikström *et al.* 1982).

Additional microorganisms have been entrapped in various polymers and some enzymic activities tested. Cells of *Mycobacterium* sp. (ATCC 29172), entrapped in agar (soy oil at 50°C), are almost as efficient as freely suspended cells in the conversion of β -sitosterol to $\Delta^{1,4}$ -androstadiene-3,17-dione. This catalytic reaction requires a functional multi-enzyme system.

Furthermore, agarose-entrapped (paraffin oil at 37°C) cells of *Gluconobacter oxydans* (ATCC 15163) were as efficient as freely suspended cells in the conversion of sorbose to 2-ketogulonic acid, as is shown in Fig. 6. For this conversion a two-enzyme system consisting of sorbose dehydrogenase and L-sorbose oxidase is required. When the *Gluconobacter* cells are co-immobilized with cells of *Pseudomonas putida* (ATCC 21812), containing high activities of the second enzyme, L-sorbose oxidase, an increased productivity of 2-ketogulonic acid is observed (Fig. 6). The observed increased conversion rate by the co-immobilized cells is probably

due to a higher sorbosone concentration present in the microenvironment of the gel-entrapped cells. This would be an analogy to the increased rates obtained with immobilized multi-enzyme system (Srere *et al.* 1973, Siegbahn and Mosbach, 1982). The viability of these immobilized microorganisms has not been investigated but it is highly likely that these cells also are fully viable. In this context, it should be mentioned that an efficient conversion of sorbose to 2-ketogutonic acid has been achieved with coimmobilized cells of *Pseudomonas syringae* and *Gluconobacter melanogenus* (Martin and Perlman 1976). In this study the cells were entrapped in polyacrylamide.

We have also developed a technique allowing for the entrapment of sensitive animal cells. This technique differs somewhat from the method used for other cell-types in that the gel is produced enzymatically. The cells are first mixed with fibrinogen and thrombin is then added to this suspension which is subsequently transferred quickly to paraffin oil at room temperature. As the enzyme converts the fibrinogen to fibrin, a solidification occurs and beads are formed. The erythroleukemic cell line K-562 appears to be unaffected after immobilization by this procedure and cell division occurs already after one day. After 5-6 days incubation, small colonies have developed, as shown in Fig. 7. Since these colonies are evenly distributed throughout the beads, there is no significant limitation of nutrients within the fibrin beads. K-562 cells normally grow in suspension and they do not require a surface to grow on, as do many other so called "anchorage-dependent" animal cells. We have also immobilized anchorage-dependent cell lines, e.g. W 49 (a rat colon carcinoma cell line), by entrapment in fibrin. The required growth surface is apparently supplied by the fibrin-fibres within the beads. Thus, both types of animal cells can be immobilized viably with the method described here.

Finally, we also prepared alginate beads in a two-phase system using a slightly modified procedure. The hydrophobic phase, in this case, contains an emulgator. When the alginate-cell suspension is stirred in this phase an emulsion is formed which subsequently is transferred into a Ca^{2+} -containing solution. After stabilization of the beads for 15 min they can be collected and washed. When adjusting the stirring speed while preparing the emulsion, beads with a diameter of down to 10 μm can be obtained. This may be advantageous in some cases, since the conventional method of entrapping cells in alginate leads to preparations exerting diffusion hindrance.

In conclusion, the method, described in this paper, for entrapping various cells with preserved viability appears to be generally applicable. Microbial, algal, plant and animal cells have been immobilized in various matrices without any significant loss of their biosynthetic capacities or viability, as long as the polymer used gels under mild conditions (e.g. low gelling temperature or enzymatic gel formation). Additional advantages are that the bead size can easily be regulated by adjusting the stirring speed during preparation. The procedure appears to be relatively easy to scale up.

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Table 1. Conditions for entrapment of various cells in different matrices. General conditions are given in the experimental section.

Species	polymer	final polymer concentration (%)	final cell concentration (%)	immobilization temperature (°C)	hydrophobic phase
<i>Saccharomyces cerevisiae</i>	agarose	2	25	50	soy oil
	carrageenan	2	25	37	soy oil
	polyacrylamide	14	20	20	soy oil
<i>Mycobacterium</i> sp.	agarose	3	10	40	paraffin oil
<i>Gluconobacter oxydans</i>	agarose	3	5	40	paraffin oil
<i>Pseudomonas putida</i>	agarose	3	5	40	paraffin oil
<i>Glycine max</i> (plant cell)	agar	3	10	45	various cf. Table 3
	agarose	2,7	10	45	soy oil
	agar	4.5	10	45	soy oil
	carrageenan	2,7	10	45	soy oil
	alginate	4,5	10	20	soy oil
K-562 (animal cell)	fibrin	1	5 10^5 cells/g gel	37	paraffin oil

Table 2. Conditions for HPLC-assays.

Parameter	Ethanol	Steroids	2-Ketogulonic acid
Column size	200 x 4.6 mm	250 x 3.1 mm	200 x 4.6 mm
Column packing material	Nucleosile-C ₁₈	Lichrosorb SI 60 (5 µm)	Nucleosile-C ₁₈ (5 µm)
Mobile phase	H ₂ O	heptan:ethanol (98:2)	0.01 M Tris-HCl buffer pH 7.5 + 0.05 % tetra- butylammoniumbromide
Flow rate (ml/min)	1.0	5	0.5
Detection	RI	UV 210 nm	RI

Table 3. Relative respiration of entrapped plant and yeast cells after immobilization in various hydrophobic phases.

Hydrophobic phase	relative respiration (%)	
	plant cells ⁺	yeast cells ⁺⁺
soy oil	100	100
paraffin oil	91	n.d.
silicon oil	100	n.d.
tri-n-butylphospahte	100	n.d.
dibutylphtalate	82	n.d.
toluene:chloroform (73:27 + Arlace1 83 (2 % w/v)	0	7

⁺ soybean cells (10 % w/w) in agar (3 % w/v)

⁺⁺ yeast cells (20 % w/w) in polyacrylamide (14 %)

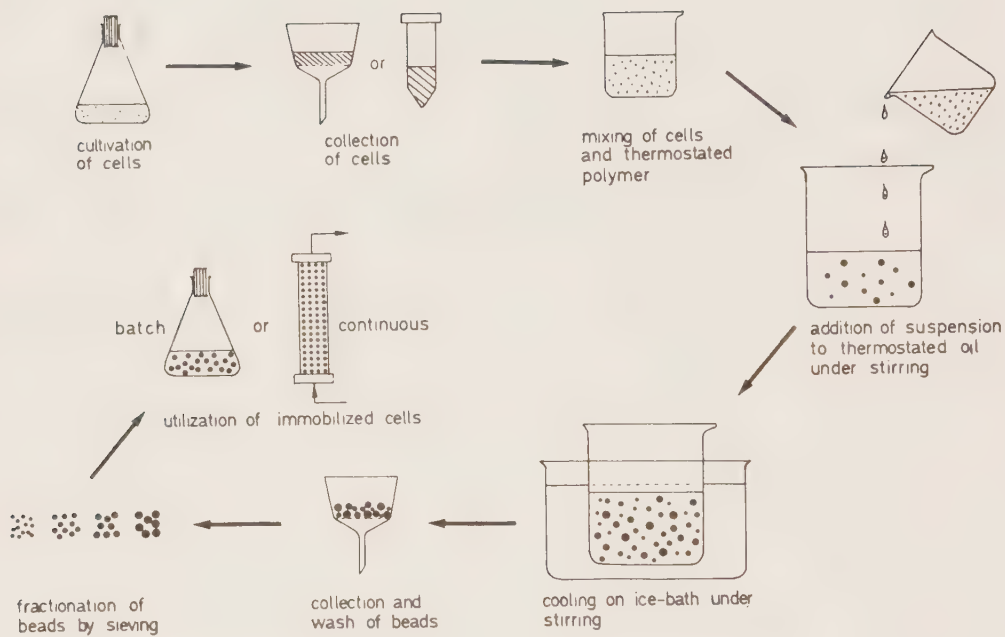
n.d. = not determined

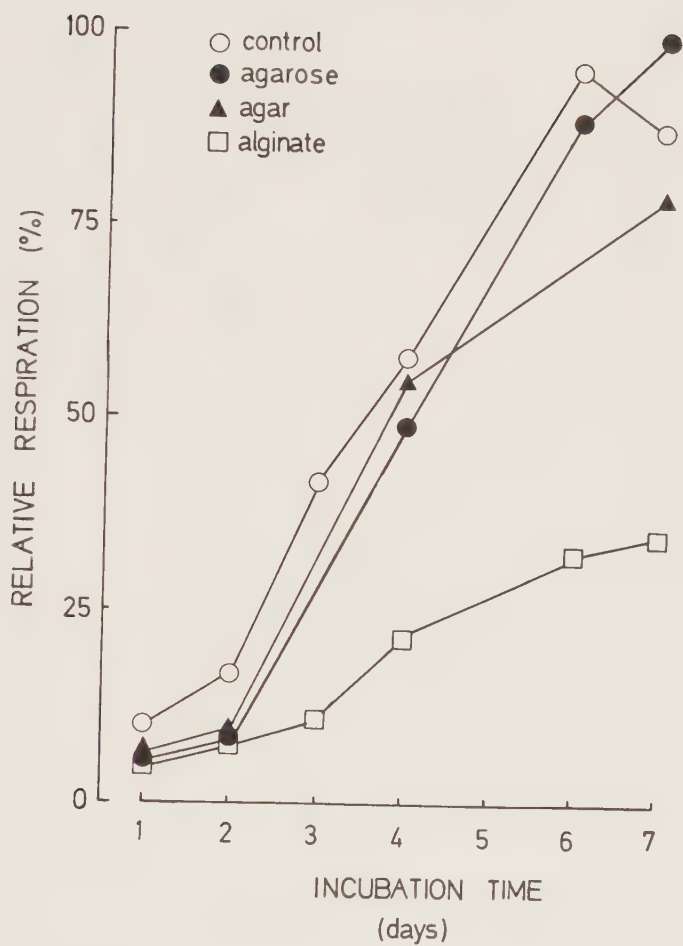
Table 4. Fermentation capacity of various preparations of immobilized yeast cells before and after *in situ* activation with nutrient media.

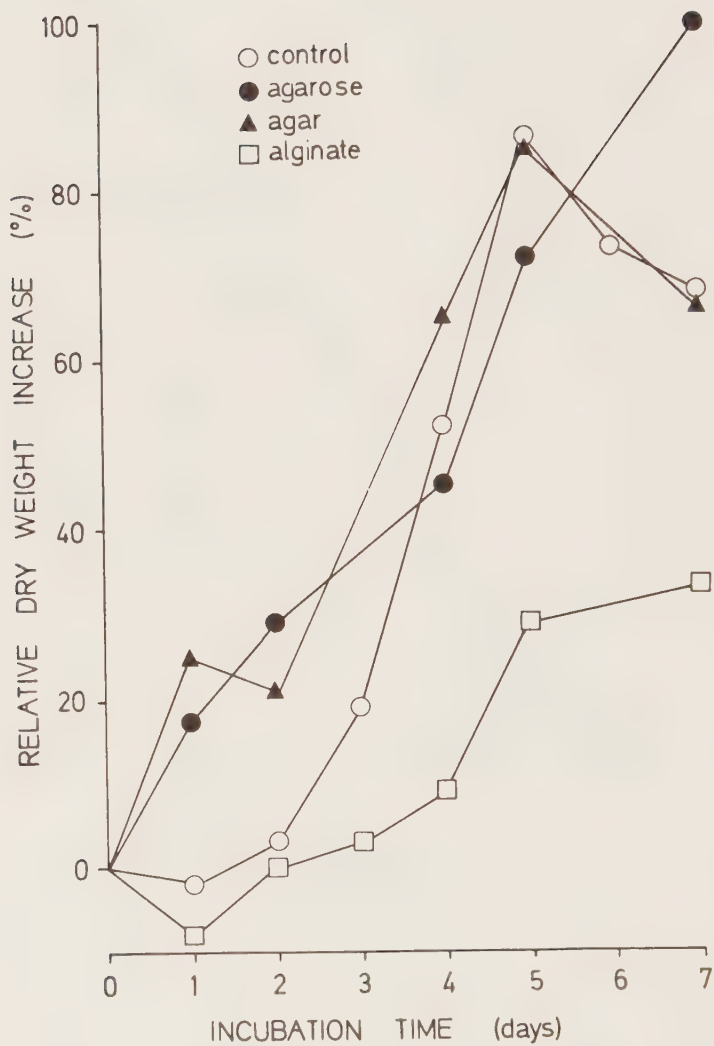
Preparation	Ethanol production (mmol/h g wet beads)	
	before activation	after activation
alginate	0.05	8.0
agarose	0.05	4.0
carrageenan	0.05	4.5

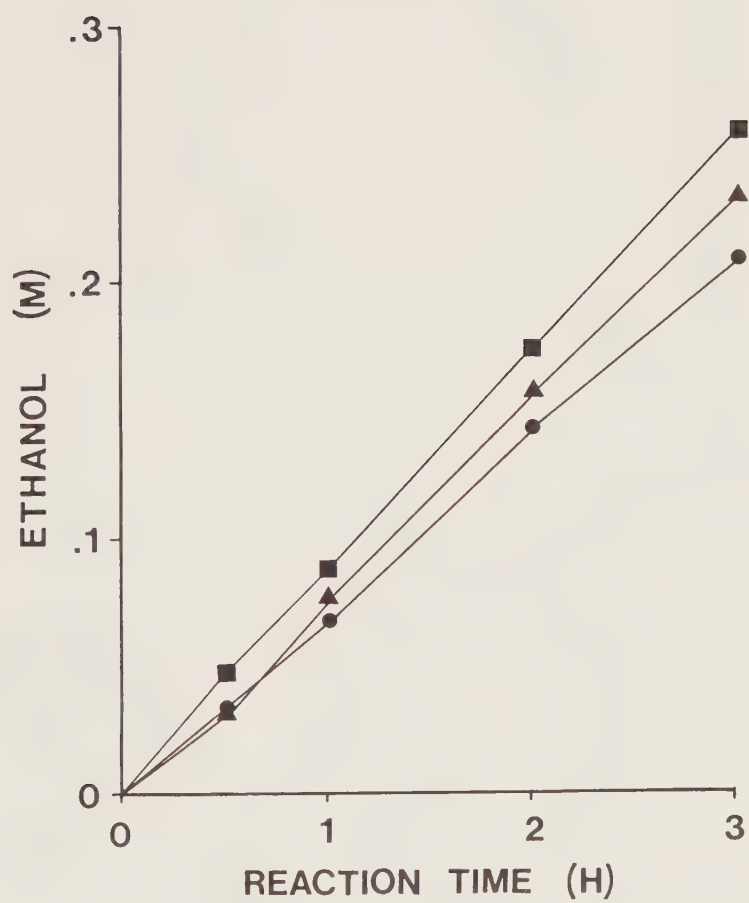
FIGURE LEGENDS

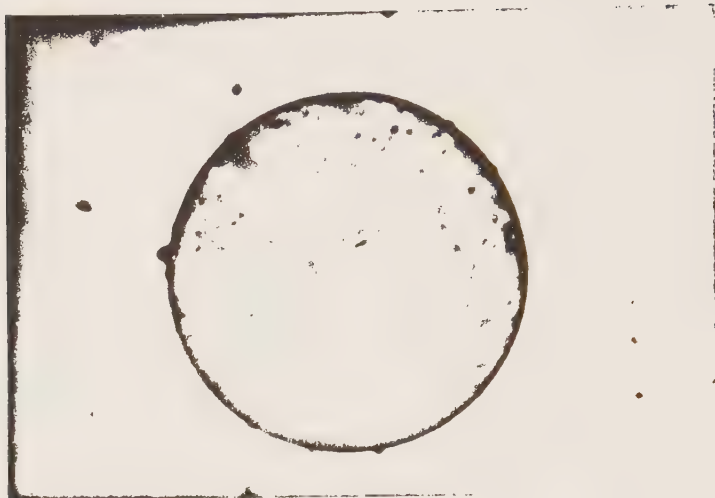
- Figure 1. Schematic diagram of the procedure leading to entrapped viable cells using various matrices in a two-phase system. In the example shown, the beads are formed by cooling of a warm agarose-cell suspension after suspension in oil.
- Figure 2. Relative respiration of various preparations of immobilized cells of *Glycine max* (soybean) as a function of incubation time. The cell preparations were incubated in the growth medium as described in Materials and Methods. Control = freely suspended cells.
- Figure 3. Relative dry weight of various preparations of immobilized cells of *Glycine max* (soybean) as a function of incubation time. The cell preparations were incubated in the growth medium as described in Materials and Methods. Control = freely suspended cells.
- Figure 4. Fermentation capacity of various preparations of immobilized *Saccharomyces cerevisiae* cells. Beads (5 g wet weight) containing 25 % cells (w/v) were incubated in 0.25 M glucose (20 ml) at 30°C. At the intervals indicated aliquots (100 µl) were taken and analyzed by HPLC. (■) alginate; (●) carrageenan; (▲) agarose.
- Figure 5. Micrographs of agarose-entrapped yeast cells (A) Freshly prepared bead and (B) bead after incubation in nutrient medium for 12 h. The cells were stained with Fuchsin as described in Materials and Methods.
- Figure 6. Production of 2-ketogulonic acid from sorbose by various preparations of microbial cells. Free cells (open symbols) (0.25 g, wet weight) or agarose entrapped cells (solid symbols) (5 g beads; 10 % w/v cells) were incubated in medium (100 ml) containing 3 % sorbose and 2 % CaCO₃. (△) and (▲) *Gluconobacter oxydans*; (□) and (■) *Gluconobacter oxydans* + *Pseudomonas putida* (10:1).
- Figure 7. Micrograph of fibrin-entrapped K-562 cells. The cells were stained with Türk's solution as described in Materials and Methods.

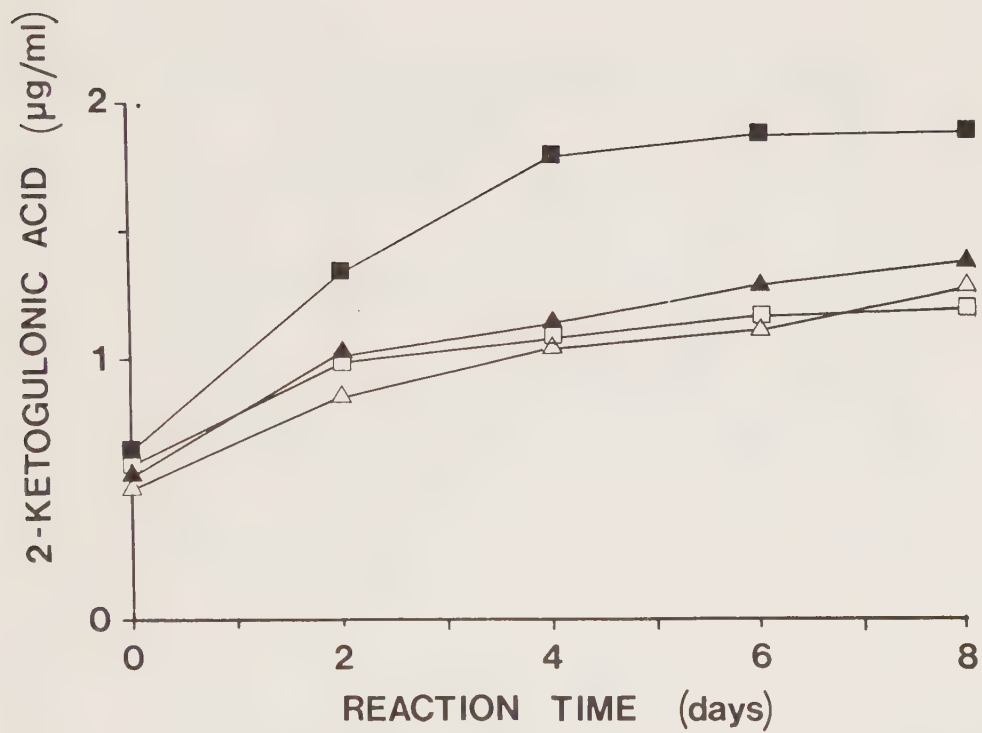


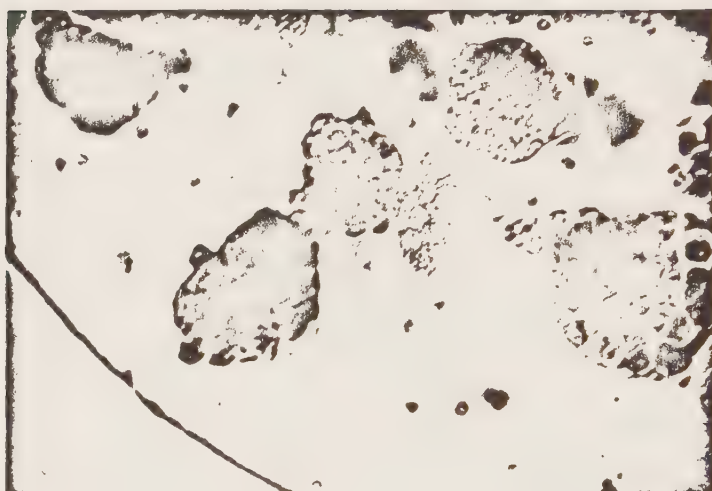












PERMEABILIZATION OF IMMOBILIZED PLANT CELLS, RESULTING IN
RELEASE OF INTRACELLULARLY STORED PRODUCTS WITH PRESERVED CELL VIABILITY

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SUMMARY

Plant cells, entrapped in agarose or alginate, were permeabilized in a new procedure that retain cell viability, permitting the possibility of reusing biomass after release of intracellular products. Dimethylsulfoxide (DMSO) was used to make the cells permeable. The activity of isocitrate dehydrogenase, an intracellular enzyme, was used as an indicator of plasma membrane permeability. Cells from three plant species require different concentrations of DMSO for complete permeabilization (i.e. for maximal isocitrate dehydrogenase activity). Cells of C. roseus permeabilized with up to 5 % DMSO remained viable, and released 85-90 % of the intracellularly stored products (ajmalicine isomers). In model production systems, C. roseus cells entrapped in agarose or alginate beads were intermittently permeabilized for release of products in a cyclic process. An increase in product yield was observed for each cycle because of increase in biomass (cell growth) within the beads.

1. INTRODUCTION

The production of commercially significant biochemicals by fermentation of plant cells is a technique of great biotechnological potential (Barz et al. 1977, Staba 1980). A wide variety of secondary products, many of which are of commercial interest, have been isolated from plant tissue cultures (Zenk 1978, Kurz and Constabel 1979). A major problem in producing such substances in a biotechnological process is, however, the low yield of the target substance. Although selected cell lines, i.e. high-producing clones, may be used to increase the yield (Ogino et al. 1978, Deus and Zenk 1982, Yamamoto et al. 1982), the generation of biomass, which is slow and expensive, limits at present the applicability of plant cell cultures on a commercial basis. Thus, it appears to be of importance to utilize the biomass over an extended period of time. We have been investigating the feasibility of using immobilized plant cells for this purpose (Brodelius et al. 1980, Brodelius and Mosbach 1982, Brodelius 1982) and have observed a production phase that is longer for immobilized plant cells than for freely suspended cells.

Products of commercial interest are often intracellular and so we have focused on methods to release products without affecting the viability and biosynthetic capacity of the immobilized cells. In an initial study (Felix et al. 1981) on the permeabilization of immobilized plant cells, we used relatively harsh methods to make cells permeable that resulted in cell death. In this paper we report on a method for reversible permeabilization of immobilized plant cells, that means render cells permeable for the release of intracellular products without destroying cell viability and permitting the reuse of biomass

after product isolation. This method may be of fundamental importance for the development of a economically feasible process for the production of biochemicals with cultivated plant cells.

2. MATERIALS AND METHODS

2.1. Chemicals

Agarose (type VII), NADP, and D,L-isocitric acid (trisodium salt) were obtained from Sigma (St. Louis, MO.). Sodium alginate (Manucol DH) was from Alginate Industries (Girven, UK). Tryptamine and DC-Alufolien (Kieselgel 60 F₂₅₄) were obtained from Merck (Darmstadt, FRG) and (2-¹⁴C)-tryptamine from NEN (Boston, MA.). Ajmalicine was purchased from Roth (Karlsruhe, FRG). Secologanin was kindly provided by Dr. C.R. Hutchinson, University of Wisconsin, Madison, WI. All other chemicals and biochemicals were of analytical grade and were purchased from commercial sources.

2.2. Cultivation of cells

Plant cells were cultivated at 26 °C in suspensions on a rotary shaker at 100 rpm in the following media:

Catharanthus roseus: LS-medium (Linsmaier and Skoog 1965) supplemented with 2,4-dichlorophenoxyacetic acid (2.2 mg/l) and naphthaleneacetic acid (1.86 mg/l), pH 6.0 (medium A).

Daucus carota Ca68 (this cell line was kindly supplied by Dr. I.A. Veliky,

National Research Council Canada, Ottawa): 7lv-medium (Veliky and Rose 1973) supplemented with 2,4-dichlorophenoxyacetic acid (1.5 mg/l), indoleacetic acid (1.0 mg/l), naphthaleneacetic acid (0.1 mg/l) and kinetin (0.25 mg/l), pH 5.0 (medium B).

Datura innoxia: SH-medium (Schenk and Hildebrandt 1972) supplemented with 2,4-dichlorophenoxyacetic acid (0.5 mg/l), kinetin (0.1 mg/l) and p-chlorophenoxyacetic acid (2.0 mg/l), pH 5.8 (medium C).

2.3. Immobilization of plant cells

Cells of C. roseus, D. carota and D. innoxia were immobilized by entrapment in agarose according to a newly developed procedure (Nilsson et al. 1982). The cells (2.0 g wet weight) were mixed with agarose (8 g of a 5 % w/v solution) at 40 °C. The suspension was subsequently poured into soy oil (50 ml) also kept at 40 °C under continuous stirring. When agarose droplets of appropriate size (diameter approximately 1 mm) had formed, the whole mixture was cooled on an ice bath until the agarose solidified. The beads containing the immobilized cells were collected and washed.

Cells of C. roseus were also immobilized by entrapment in calcium alginate as previously described (Brodelius et al. 1979). The cells (15 g wet weight) were mixed with sodium alginate (35 g of a 5 % w/v solution) and the suspension dripped into medium A containing 50 mM CaCl_2 (200 ml). After 30 min the beads were collected and washed with medium A containing 5 mM CaCl_2 .

2.4. Permeabilization procedure

Freely suspended or immobilized cells were permeabilized by incubation in medium A containing various concentrations of DMSO on a rotary shaker (100 rpm) for 30 min. A 10:1 ratio of medium to beads was used unless otherwise stated.

2.5. Biosynthetic studies

The synthesis of ajmalicine isomers from tryptamine and secologanin by agarose- and alginate-entrapped cells of C. roseus with intermittent product release was studied over a period of 12 to 14 days (see Fig. 6 and Fig. 7) The growth phase was carried out by incubating the immobilized cells (2.0 g agarose or 5.0 g alginate beads) in medium A (10 ml). Before transfer to the production phase, the beads were washed (1 x 10 ml) with a hormone-free "alkaloid-producing" medium (Zenk et al. 1977) (medium D). The beads were then transferred to medium D (5 ml) containing ^{14}C -labelled tryptamine (0.5 mM) (350,000 dpm/pmol) and secologanin (0.5 mM). After 3 days the beads were collected and washed with medium D (2 x 10 ml) and then placed in medium D (10 ml) containing 5 % (v/v) DMSO for 30 min. The DMSO-containing medium was then removed, and the beads were washed with medium D (2 x 10 ml) and transferred to medium A (10 ml). The growth/production cycle was then repeated with subsequent permeabilization. The media collected were combined, adjusted to pH 10 and extracted with methylene chloride (3 x 10 ml). The extracts were analyzed for radiolabelled ajmalicine by TLC-chromatography (Brodelius and Nilsson 1980).

2.6. Analytical procedures

Isocitrate dehydrogenase activity within immobilized permeabilized plant cells was determined as has been described (Felix et al. 1981).

Cell growth was determined by following the increase in dry weight as a function of incubation time. Samples were collected on preweighed dry filter papers and dried at 60 °C until constant weight.

3. RESULTS AND DISCUSSION

3.1. Monitoring of permeability

Various enzyme activities within plant cells can be employed for monitoring the permeability of the plasma membrane. Enzymes requiring nucleotide coenzymes such as NADP, ATP or CoA are convenient to use for this purpose. These coenzymes cannot penetrate an intact plasma membrane and thus no enzyme activity is expressed unless the plasma membrane of the cell has been made permeable. In our initial studies (Felix et al. 1981) on the permeabilization of immobilized plant cells, we investigated a number of enzymes, including hexokinase, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, malic enzyme, and citrate synthase, and found essentially no enzyme activity unless the cells were permeabilized.

In the present study we have used isocitrate dehydrogenase (ICDH) to monitor the permeability of the plasma membrane. The immobilized cell preparations were incubated in a solution containing the substrate with constant stirring. The supernatant was recirculated with a peristaltic pump through a flow cuvette in a spectrophotometer and the absorbance at 340 nm was recorded con-

tinuously. The observed changes in NADPH concentration over time are illustrated in Fig. 1 for untreated and DMSO-treated cells of C. roseus. Untreated cells show a relatively low ICDH-activity, while cells treated with DMSO for 30 min express a high activity. This indicates that DMSO has rendered the cells permeable to isocitrate and NADP(H), allowing the direct assay of an intracellular enzyme.

3.2. Effect of DMSO concentration on permeability and viability

A wide variety of substances can be employed for the permeabilization of immobilized plant cells (Felix et al. 1981). DMSO was selected as the permeabilizing agent in this study since treatment with this substance results in preparations with relatively high enzyme activity expressed (Felix et al. 1981, Felix and Mosbach 1982). Furthermore, DMSO is widely used to pretreat cells (both plant and animal) before freezing (cryopreservation) in order to lower the detrimental effect of ice crystals (Dougall and Wetherell 1974, Bajaj 1976). From cryopreservation experiments it can be concluded that the cells remain viable after treatment with DMSO.

In order to determine the optimal time for permeabilization, immobilized cells of C. roseus were treated with 5 % DMSO for various periods of time with subsequent assay of ICDH activity. The immobilized cells appeared to be completely permeabilized (maximal ICDH-activity reached) after treatment for 5 min as shown in Fig. 2. The immobilized cell preparations were, however, usually treated for 30 min in order to assure maximum permeabilization.

The effect of DMSO concentration on the permeability of the plasma membrane

of various immobilized plant species was investigated. Fig. 3 shows that the sensitivity of the plasma membrane to DMSO differs considerably for the three species studied. Cells of C. roseus appear to be completely permeabilized after treatment with 5 % DMSO, while cells of D. carota and D. innoxia require around 10 % and 25 % DMSO, respectively, for complete permeabilization. These differences may be due to differences in the morphology of the cell aggregates or in the composition of the plasma membranes and/or cell walls.

The lowest concentration of DMSO required for full permeabilization can easily be estimated from experiments of this type. It is of importance to determine this concentration, i.e. the mildest conditions for maximal effect, since it can be expected that the viability of the treated cells is highly influenced by the DMSO concentration used. To verify this, suspensions of C. roseus cells were treated with various concentrations of DMSO, washed free of the DMSO and incubated in growth medium (medium A). The increase in dry weight as a function of incubation time is shown in Fig. 4. The cells treated with 2 % DMSO grow at approximately the same rate as untreated cells. On the other hand, the cells treated with 5 or 10 % DMSO show a lag phase of approximately 2 and 8 days, respectively, before growth occurs. Catharanthus cells can apparently tolerate treatment with up to 10 % DMSO for 30 min without loss of viability, and it appears possible to permeabilize the plant cells while preserving viability. Whether the long lag phase observed for the cells treated with 10 % DMSO is due to survival of only a few cells or to slow recovery of the treated cells has not been determined. In this context, it should be pointed out that a relatively high cell density is required for the survival of plant cells in suspension after transfer to a fresh medium because the medium has to

be conditioned by the cells, which argues for the survival of a relatively large number of cells also after treatment with 10 % DMSO.

The approach for establishing the minimal concentration of DMSO required for maximal effect as outlined above may also find applications in cryopreservation experiments to increase the survival rate of preserved cells. It appears that the concentration of DMSO used in such experiments has not been optimized. In one study, it was concluded that for growth of carrot cells after freezing and thawing, 5 or 10 % DMSO was superior to 0 or 15 % DMSO (Dougall and Wetherell 1974). These results are in agreement with our findings that 10 % DMSO permeabilizes carrot cells (Fig. 3).

3.3. Product release

The synthesis of the indole alkaloid ajmalicine from distant precursors was used as a model production system for plant biochemicals. Agarose-entrapped cells of C. roseus were incubated in a medium containing two precursors - tryptamine (labelled with ^{14}C) and secologanin - and after three days of incubation, the cells were analyzed for ajmalicine isomers. One sample of immobilized plant cells was made permeable to ajmalicine with a 5 % DMSO treatment in medium D and a parallel sample was extracted with methanol in a Soxhlet apparatus for total ajmalicine. In the DMSO treated cells, 85-90 % of the total intracellular ajmalicine was released due to permeabilization of the entrapped cells. Quantitative release of products is thus possible under conditions where the viability of the immobilized cells is not affected to any great extent.

3.4. Intermittant release of products: A model production system for plant cell biochemicals

The results of the experiments discussed above suggest, in principle, that it would be possible to intermittantly release intracellular products by permeabilization of the immobilized cells as schematically illustrated in Fig. 5. The cycle, including an optional growth phase, a production phase and a permeabilization procedure, may be repeated several times.

We have carried out some model experiments according to the procedure outlined in Fig. 5. The synthesis of ajmalicine isomers from tryptamine and secologanin was studied with immobilized cells of C. roseus. The results have been summarized in Fig. 6 and Fig. 7. Cells entrapped in agarose (Fig. 6) or calcium alginate (Fig. 7) could intermittantly be treated with DMSO to release the produced ajmalicine isomers. No significant difference was observed between agarose- and alginate-entrapped cells in these biosynthetic studies. A hormone-free medium was used in the production phase in order to limit growth. The increase in product yield observed in both experiments was most likely due to the increase of biomass due to the growth of cells within the polymer beads. Growth occurred not only in the growth medium (medium A) but also to some extent in the production medium (medium D). For a steady state production optimization of the cycle has to be carried out. At present such studies are being carried out in our laboratory. The extension to de novo synthesis of secondary products from a simple carbon source with subsequent release by permeabilization is also in progress.

4. CONCLUSIONS

The results presented in this report clearly demonstrate that it is possible to release products stored within immobilized cells by a permeabilization procedure that does not affect the viability and biosynthetic capacity of the cells. A continuous process based on intermittent release of products has been developed that can be of fundamental importance for the future development of the production of biochemicals with cultivated plant cells since the biomass can be reutilized in such a process.

ACKNOWLEDGEMENT

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FIGURE LEGENDS

FIGURE 1. Time course of the isocitrate dehydrogenase reaction in agarose-entrapped cells of C. roseus. The reaction mixture (5 ml substrate solution plus 1 g of wet beads) was monitored continuously at 340 nm by pumping the supernatant through a flow cell at a rate of 5 ml/min.

FIGURE 2. Relative isocitrate dehydrogenase (ICDH) activity as a function of permeabilization time. 100 % ICDH activity is defined as the maximal amount of enzyme activity observed. Agarose-entrapped cells of C. roseus (1 g of wet beads) were treated with 5 % DMSO in medium A for the times indicated and then washed rapidly and assayed as described in the legend to Fig. 1.

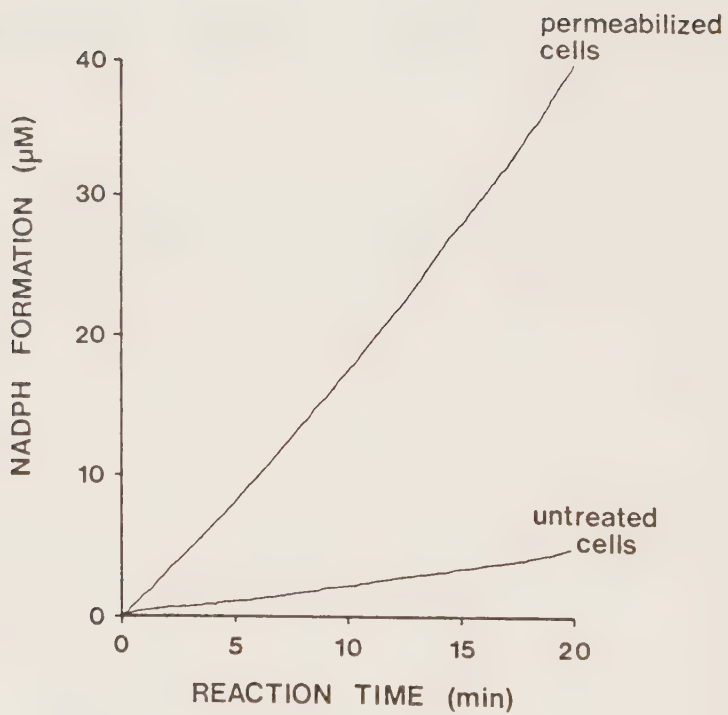
FIGURE 3. Relative isocitrate dehydrogenase activity as a function of DMSO concentration. 100 % ICDH activity is defined as the maximal amount of enzyme activity observed for each cell preparation. Beads (1 g wet weight) were treated with the DMSO concentrations indicated in medium A for 30 min then washed and assayed as described in the legend to Fig. 1. (—●—) C. roseus, (—■—) D. carota, (—▲—) D. innoxia.

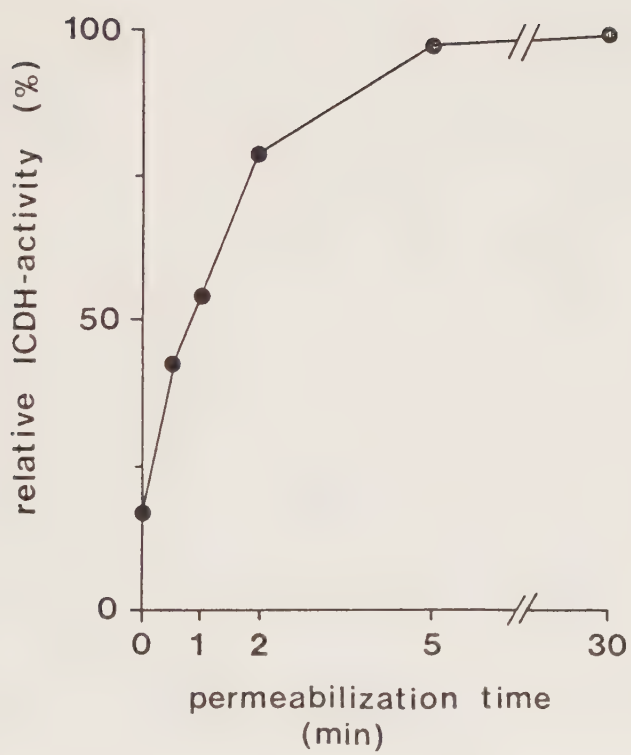
FIGURE 4. Dry weight of various preparations of freely suspended cells of C. roseus as a function of incubation time. The cells were treated with the DMSO concentration indicated for 30 min before inoculation into growth medium (medium A).

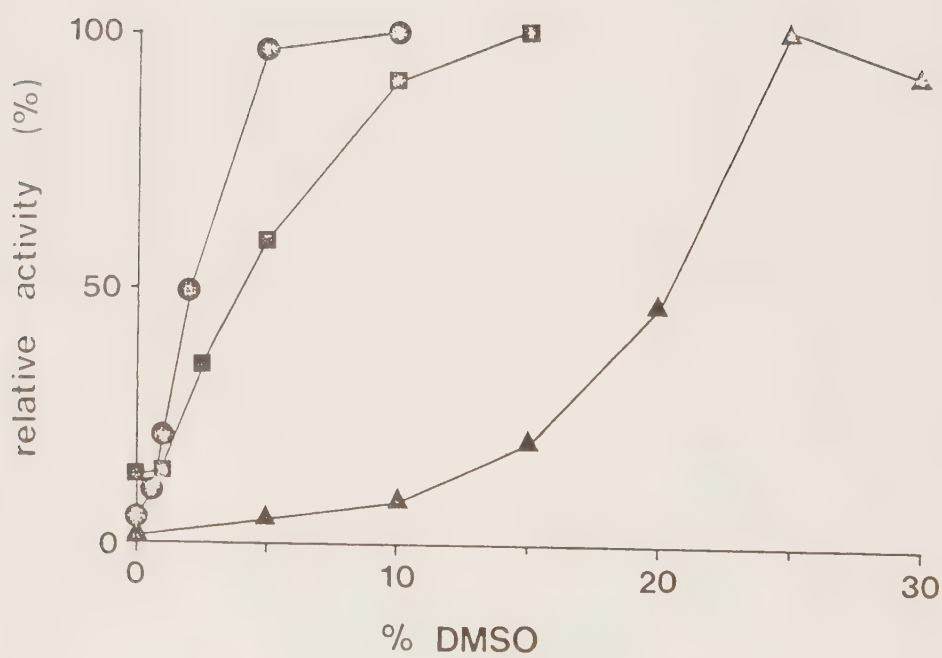
FIGURE 5. Schematic diagram of a process for the production of intracellular plant biochemicals using intermittent product release from immobilized plant cells.

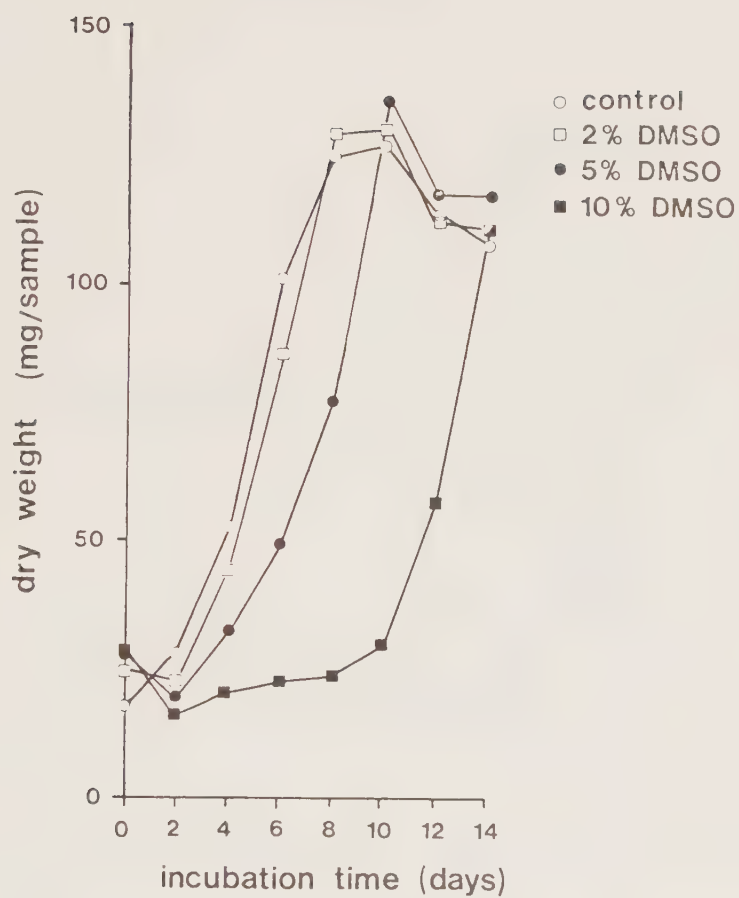
FIGURE 6. Ajmalicine production by agarose-entrapped cells of C. roseus. The cells were intermittantly permeabilized (arrows) as described in the methods section.

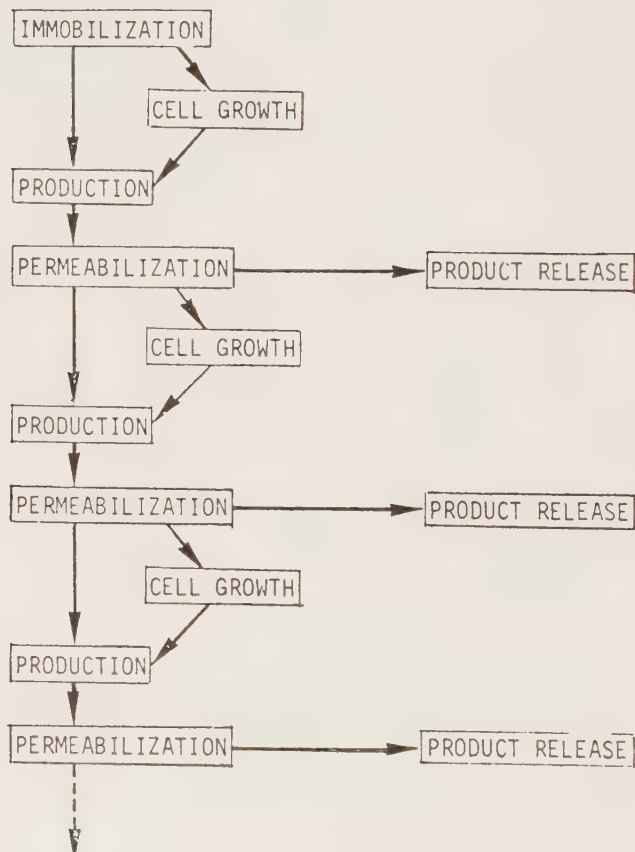
FIGURE 7. Ajmalicine production by alginate-entrapped cells of C. roseus. The cells were intermittantly permeabilized (arrows) as described in the methods section.

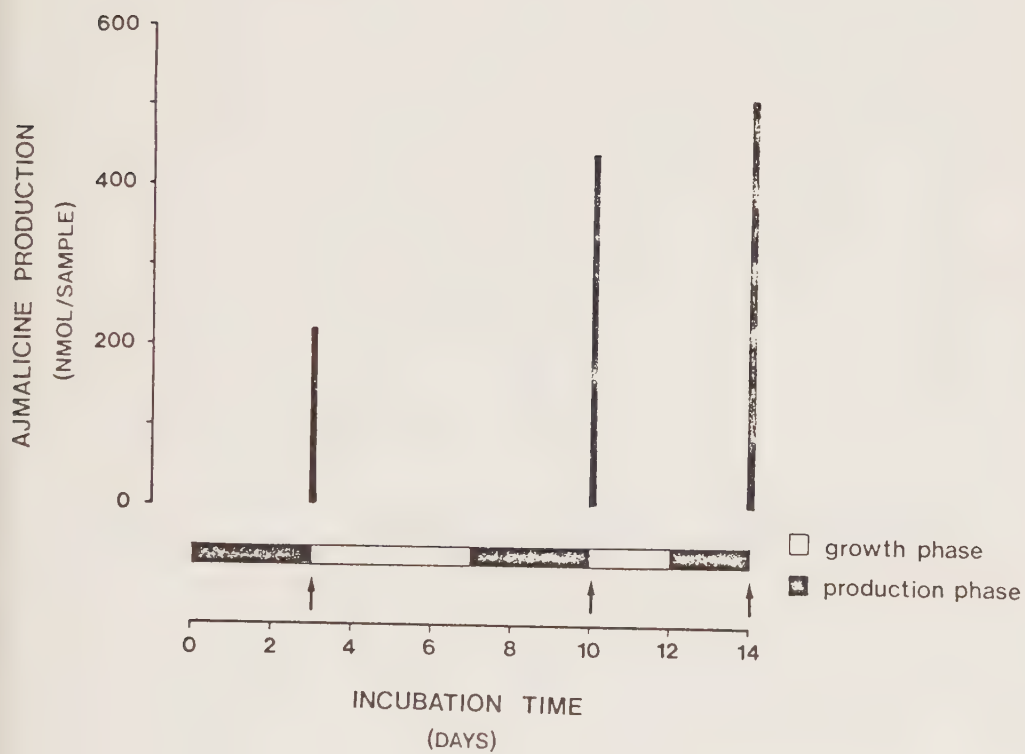


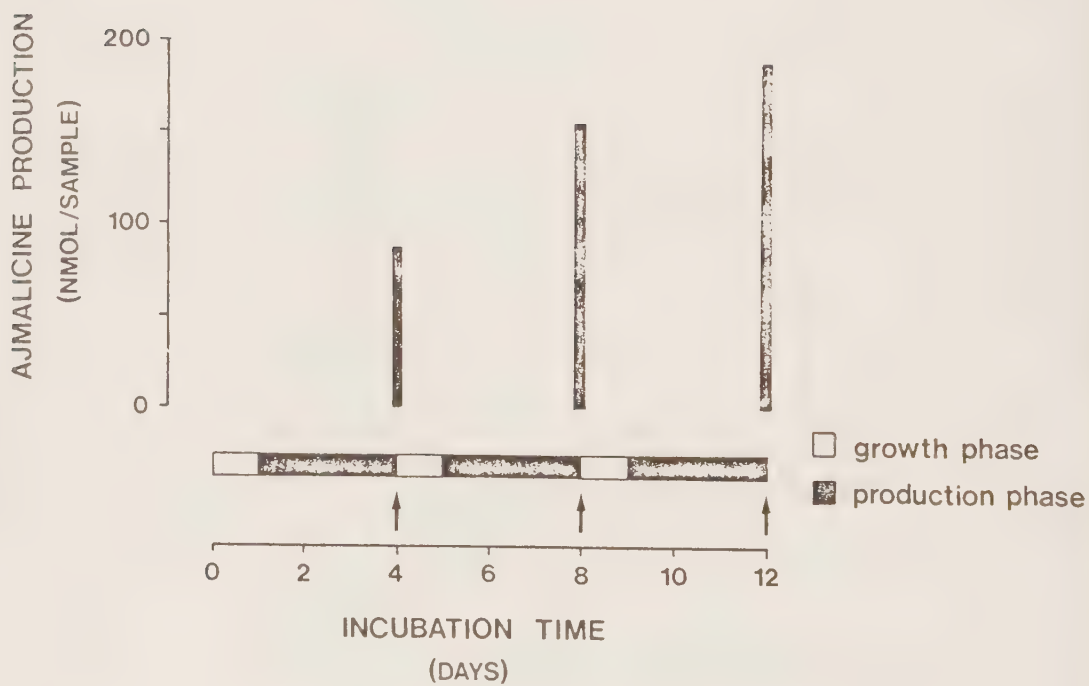














ENTRAPMENT OF ANIMAL CELLS FOR THE PRODUCTION OF MONOCLONAL ANTIBODIES
AND OTHER BIOMOLECULES

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Animal cell technology is finding increasing interest, notably because of the capacity of animal cell cultures to synthesize or transform complex compounds such as virus vaccines, immunochemicals, hormones or enzymes¹. For the growth of surface-dependent cells, microcarrier technology is gaining in importance². In analogy hereto, we have attempted to immobilise surface independent cells, normally grown in suspension, by entrapping them in polymer microbeads. These studies were carried out since such entrapment would give increased stability to the normally fragile animal cells, allow for high cell densities to be achieved within the beads and make such preparations suitable for continuous operation. At the same time, the need for separation of the desired product from the cells is obviated. With the model systems studied, we were able to show that hybridoma, as well as other cell lines entrapped in agarose microbeads, remained viable. Both immunoglobulins and lymphokines (IL2) were exported through the microbeads into the medium for 1-3 weeks, at levels corresponding well to those produced with free cells.

In previous studies it had been demonstrated that animal cells can be entrapped in a viable state in alginate beads, leading to preparations in which the cells are either embedded throughout the network of the bead³ or are kept within the microcapsules, which are characterized by a shell of alginate-polyethyleneimine⁴. A drawback of this technique, when applied to animal cells, is the requirement for positive counter ions in order to keep the polymeric network intact, a requirement that may adversely affect viability and growth of animal cells; and/or the fragile nature of such preparations. Furthermore, a number of larger biomolecules, especially monoclonal antibodies,

cannot diffuse out through the polymeric network, a fact which will prevent the use of such preparations for continuous processes. We wish to report on an alternative technique for circumventing these problems whereby the animal cells are entrapped in agarose beads, the latter formed on cooling a cell-agarose suspension in oil medium⁵. By using oil such as paraffin as a suspension medium, the cells are not harmed, in contrast to normally applied organic solvent media⁶. When applying this technique, agarose microbeads, usually made up of 2.5% agarose, were prepared with an average size of 80-100 μm . As seen from Fig. 1, entrapped mouse/mouse hybridoma cells are evenly distributed within the agarose network. After one week's incubation, clusters of cells appeared in the beads due to cell growth.

The same entrapped hybridoma cells were also tested for their capacity to form monoclonal antibodies $\text{IgG}_{2\text{A}}-\text{K}$ against herpes simplex type 2 glycoprotein. As seen in Table 1, daily removal and assay of the supernatant for a period of one week showed a more or less unchanged production capacity, comparable to that of free cells grown as suspension cultures in bottles. It should be noted that the two kinds of experiments refer to cultivation of cells in spinner flasks (using 2.5% agarose beads) and in roller flasks (using 1.2% agarose beads) and are thus not quite comparable. A number of other hybridoma cell lines tested, such as human/mouse hybridoma forming IgG_1-K against anti-human influenza A nucleoprotein, could also be conveniently entrapped with no impairment of their monoclonal antibody formation capacity.

A cell line producing interleukin 2, known to stimulate lymphocyte growth, was tested as a model system for the production of other biomolecules of medicinal interest. The gibbon lymphoblastoid cell line in question had a high spontaneous release of interleukin 2⁷. As is

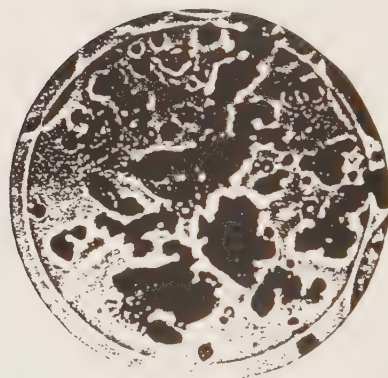
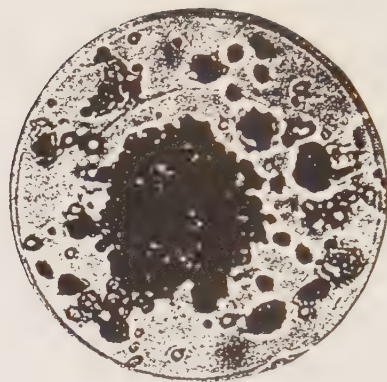
seen in Fig. 2, continuous production of interleukin 2 took place over the period of 13 days tested, when the immobilised cells were kept in spinner flasks. In this experiment a high cell concentration (2×10^7 cells/ml beads) was chosen. With conventionally grown lymphoblastoid cells in spinner flasks, such high cell densities cannot be obtained, which shows that by employing the agarose entrapment techniques, desired cell densities can be obtained artificially. This may be a great advantage since it allows the construction of efficient systems which can produce higher concentrations of the desired biomolecules in question than found with conventional suspension cultures or bottle flasks. In addition, as demonstrated for hybridoma cell lines (Fig. 1), the entrapped cells may subsequently be brought to grow further within the microbeads.

Summarizing, we feel that the here for the first time described successful entrapment of animal cells leading to preparations that can be used in continuous stirred batch reactors or columns and that continuously excrete products should be of considerable biotechnological interest.

We wish to acknowledge partial support (80-3595) by the Swedish Technical Board of Development.

Legend to Fig. 1

Entrapped hybridoma cells, LSP21, 6×10^6 cells/ml of beads. (A = 0 days; B,C = 6 days). The bead size is approximately 80-200 μm . Agarose (Sigma No. A 4018) in PBS without Ca^{2+} and Mg^{2+} at a concentration of 5%, was autoclaved and stored at room temperature. Before use the gel is melted at 70°C , then cooled to 40°C and mixed with the cells suspended in an appropriate volume of growth medium. The mixture is poured into a 250 ml round-bottom glass centrifuge tube containing an equal volume of paraffin oil (No. 7174, Merck, Darmstadt, FRG) previously washed with PBS and autoclaved. The liquids are emulsified at room temperature with a vibromixer E1 fitted with a vibrator plate P1, 54 mm diameter (Chemap AG, Männedorf, Switzerland), to the desired bead size. Then the mixing vessel is cooled in an ice bath for 5 minutes and 50 ml of growth medium is added. After another 10 minutes at room temperature, the tube is centrifuged at $1200 \times g$ for 10 minutes. The oil phase is removed by suction and approximately 100 ml of growth medium is added. After mixing with a magnetic stirrer or with the vibrator plate, the suspension is recentrifuged and the remaining oil removed. The microbeads are transferred to the cultivation vessel and further processed.



Legend to Fig. 2

Continuous production of interleukin 2 (IL2) by entrapped lymphoblastoid MLA 144 cells, which were entrapped yielding 2×10^7 cells per ml 2.5% agarose beads. 6 ml of the beads were cultivated in a 500 ml spinner vessel and agitated at 100 rpm in 200 ml growth medium, RPM 1 1640, supplemented with 5% heat inactivated foetal calf serum. About 80% of the medium was replaced daily by sedimentation and decantation. The average titer of the bottle cultures was about 1500. The IL2 assay is based on an indicator cell line which is absolutely dependent on the presence of IL2⁸. The rate of growth is dependent on the concentration of IL2, without which cells die within the test period. For performing the test, 100 μ l of sample dilutions were dropped into the wells of 96-well microplates (No. 167008, Nunc, Denmark) and 100 μ l of RPM 1640 supplement with 10% foetal calf serum containing 10,000 indicator cells was added. The plates were then incubated for 2 days at 37°C in a humidified atmosphere containing 5% CO₂. After incubation, cells were fixed at 4°C by the addition of glutaraldehyde (0.2% final concentration) for 30 minutes after which the supernatant was removed. The cells were stained by the addition of 100 μ l of a freshly prepared and filtered solution of 2% Giemsa in distilled water. The staining period was 3 hours at 37°C. After this treatment, the cells were rinsed with PBS to remove excess dye. For measurement, the dye is eluted with 100 μ l of a mixture of equal volumes of ethanol and 0.1 M NaH₂PO₄. After agitation, the plates are measured directly in a Titertek Multiscan Photometer at 620 nm. Activity is evaluated using the integral of the dose response curve.

INTERLEUKIN 2 titres in the effluent

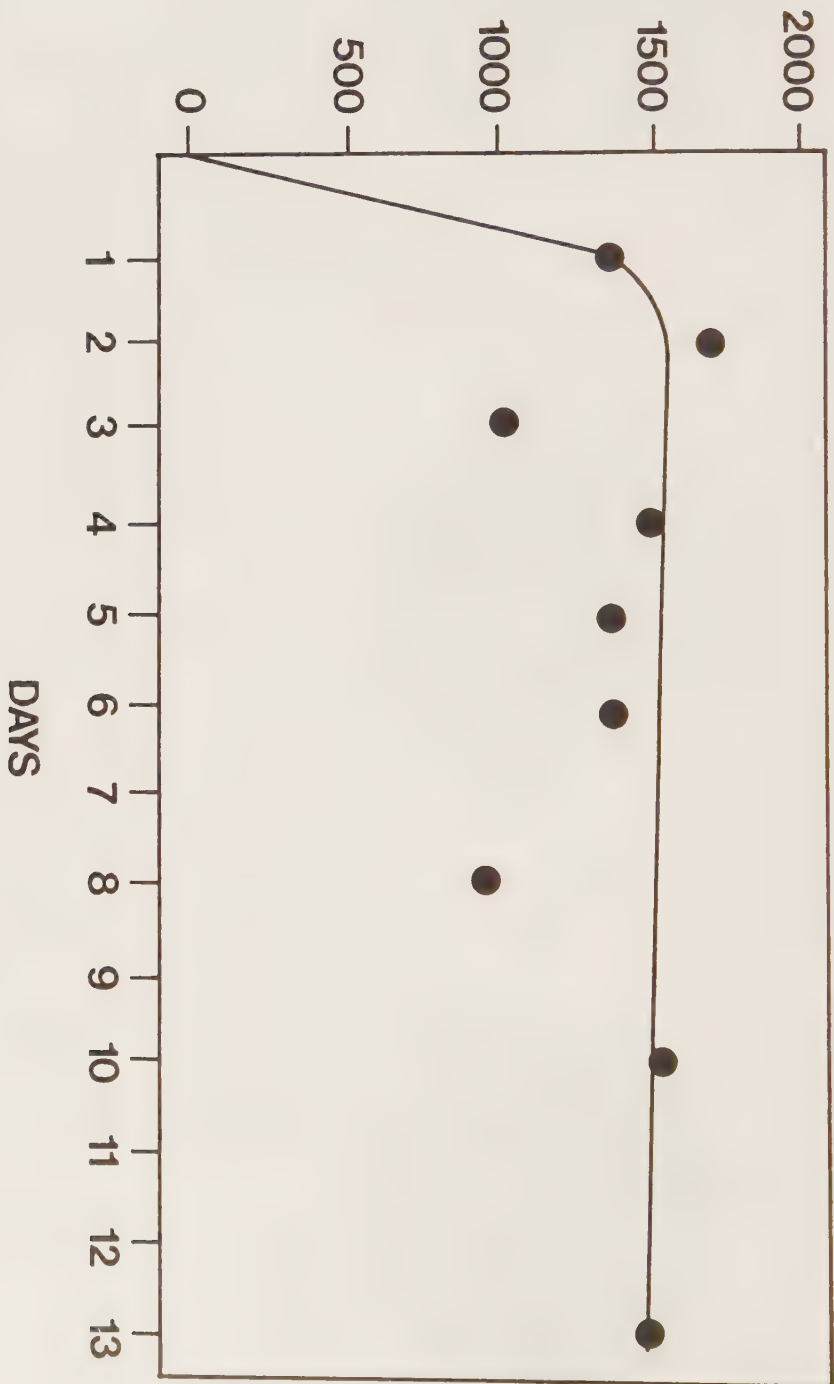


TABLE 1.

Production of IgG_{2A}-K against Herpes simplex type 2 glycoprotein by immobilized hybridoma LSP21 cells.

Day	Reciprocal Dilution	
	Expt. 1	Expt. 2
1	256	362
2	512	1024
3	256	1024
4	512	n.d.
5	512	n.d.
7	256	n.d.

Anti-herpes simplex virus (HSV) production was measured with an Elisa method. First the formed HSV antigens were allowed to bind to specific rabbit anti-HSV immunoglobulin (Dako, Copenhagen, Denmark) immobilized to Nunc immunoplates (AS Nunc, Roskilde, Denmark). In the antigen-containing wells, media or media dilutions were incubated for 30 minutes at 37°C. After washing, peroxidase conjugated anti-mouse IgG (Miles-Yeda 61-204) of 1/600 dilution was added. After further washing, the presence of peroxidase was determined by adding a staining solution containing 40 mg 1,2-phenylenediamine-dihydrochloride and 20 µl of 30% H₂O₂ per 100 ml. The reaction was stopped by adding 4 N sulphuric acid and the color which developed was measured at 492 nm. In experiment 1, LSP21 cells were entrapped yielding 6.2×10^6 cells per ml microbeads containing 2.5% agarose. 20 ml of the microbeads were cultivated in a

Legend to Table 1. cont.

250 ml spinner vessel and agitated at 100 rpm in 100 ml medium. About half of the medium (RPM 1640, supplemented with 10% heat inactivated foetal calf serum) was replaced daily by sedimentation and decantation. In Experiment 2, LSP21 cells were entrapped yielding 4.3×10^6 cells/ml of microbeads containing 1.2% agarose. Cultivation took place in plastic roller bottles (N. 3027, Falcon, Oxnard, CA, U.S.A.) each containing 50 ml of beads suspended in 250 ml of growth medium. The rolling speed was 5 rpm. 200 ml of the medium was replaced daily. The average titer of bottle cultures was 1:128.

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